

# Open Research Online

---

The Open University's repository of research publications and other research outputs

## The role of cell-mediated immunity in protection against *Bacillus anthracis*.

### Thesis

#### How to cite:

Beedham, Richard John (1999). The role of cell-mediated immunity in protection against *Bacillus anthracis*. MPhil thesis The Open University.

For guidance on citations see [FAQs](#).

© [not recorded]



<https://creativecommons.org/licenses/by-nc-nd/4.0/>

Version: Version of Record

Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.21954/ou.ro.0000ff73>

---

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

---

[oro.open.ac.uk](http://oro.open.ac.uk)

UNRESTRICTED

Richard John Beedham

Bsc (Hons)

**The Role of Cell-Mediated Immunity in  
Protection against *Bacillus anthracis***

Submitted to the Open University  
in fulfilment of the requirements for  
the degree of Master of Philosophy

Life Sciences

31 August 1999

Centre for Applied Microbiology and Research

ProQuest Number:27727935

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 27727935

Published by ProQuest LLC (2019). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code  
Microform Edition © ProQuest LLC.

ProQuest LLC.  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106 – 1346

## Abstract

Anthrax is a zoonotic disease caused by the spore-forming Gram-positive organism, *Bacillus anthracis*. Protective Antigen (PA) is the major protective component of existing licensed human vaccines. The aim of the investigation was to employ immunological techniques, experimental vaccines and animal models to shed light on the mechanism of protection against *B. anthracis* infections. It was hoped that the results would give rise to information on immune correlates of protection. This would allow a method of determining an individuals' immunological status in terms of protection against infection and provide data to assist in the development of a second-generation anthrax vaccine.

A model was used to investigate delayed-type hypersensitivity reactions and lymphocyte proliferation responses in guinea pigs immunised with different preparations. It was found that recall responses could be generated by immunisation. Memory responses are a requisite of any useful immunising preparation. However, cellular responses could not be correlated with results of an aerosol *B. anthracis* challenge. This could be due to a combination of variation within the assays and the outbred nature of the animal strain used.

The murine immune response to native and recombinant PA was investigated using different vaccine preparations and mouse strains. Lymphocyte proliferation and immunoglobulin isotyping suggested that PA produced a Th<sub>2</sub>-type response independent of the adjuvant used. Final investigation, involving passive transfer of cells or sera to animals before challenge, found that antibody was the key to protection against challenge under these conditions. This demonstrates that an antibody response is important in protection against *B. anthracis* infection.



In conclusion, investigations within this report have added to the current understanding of the mechanism of protection against *B. anthracis* infections. The results provide data for the development of an improved anthrax vaccine.

## Acknowledgements

I would like to thank everybody who has helped me over the last 6 years during the investigations at CAMR and CBD.

Dr Diane Williamson for her support and guidance. Dr Peter Turnbull for help with reagents and carrying out the *B. anthracis* challenges. Miss Kate Fowler for carrying out anthrax challenges and post-challenge counts. Dr Andy Robinson for support and encouragement. Mr Steve Eley for allowing me to continue with my MPhil although it followed me from a previous employer.

Dr Sally Sharpe for encouragement with my tentative steps at lymphocyte proliferation assays. The Biological Investigations Group at CAMR for equipment and help during my visits to their facility.

I would like to thank the CBD EAH staff for technical assistance and advice. Dr Julie Miller for endless supplies of purified recombinant PA. Mr Ken Gillhespy for his support and assistance with serology assays. Mrs Debbie Rogers for help with a number of animal investigations. Dr Mike Green for use of laboratory equipment and advice from start to finish. Miss Kate Griffin for assistance with the final passive transfer investigation. Mr Tony Stagg, Dr Rosa Taylor and Miss Caroline Redmond for encouragement and proof reading. Mr Les Baillie for support and assistance.

Finally my thanks to Mandy for just about everything! Now this is finished I can help with your proof reading !

## **Declaration**

I declare that the material contained within this thesis has not previously been submitted for a degree or other qualification, the work was performed by myself unless otherwise stated, and all sources of information have been acknowledged. The material contained within has not been published so far.

R.J. Beedham

## Contents

Abstract	i
Acknowledgments	iii
Declaration	iv
Contents	v
List of Tables	x
List of Figures	xii
1.0 Introduction	1
1.1 Classification	2
1.2 History	2
1.3 Morphology and cultivation	5
1.3.1 Persistence	6
1.3.2 Virulence factors	6
1.4 Toxin components	8
1.4.1 Protective antigen	8
1.4.2 Oedema toxin	9
1.4.3 Lethal toxin	10
1.4.4 Genetics and virulence	11
1.5 Disease in animals	12
1.6 Disease in man	12
1.6.1 Cutaneous anthrax	13
1.6.2 Intestinal anthrax	14
1.6.3 Inhalational anthrax	14
1.6.4 Anthrax meningitis	15
1.6.5 Treatment	16

1.7	Epidemiology	17
1.8	The immune system and infection	20
1.8.1	Immune response to infection	20
1.8.2	B lymphocytes and antibodies	21
1.8.3	The complement system	22
1.8.4	T lymphocytes and cell-mediated immunity	22
1.8.5	Other cells of the immune response	25
1.8.6	Vaccines	26
1.8.7	Adjuvants	28
1.8.8	Animal models	28
1.8.9	Animal models and anthrax	29
1.9	Anthrax vaccination	30
1.9.1	History	30
1.9.2	Attenuated live spore vaccine	31
1.9.3	Chemical vaccine	32
1.9.4	Efficacy problems	34
1.9.5	Immunological basis of protection	34
1.10	Aim of the investigation	37
1.11	Organisation of the investigation	38
2.0	Investigation of the cellular immune response to anthrax vaccination using a guinea pig model	39
2.1	Introduction	40
2.1.1	Anthrax investigations and guinea pig models	40
2.1.2	Delayed-type hypersensitivity	41
2.1.3	Lymphocyte proliferation assay	42

2.2	Aim of the experiments	43
2.3	Method and materials	44
2.3.1	Experimental animals	44
2.3.2	Delayed-type hypersensitivity skin test	44
2.3.3	Lymphocyte proliferation assay	46
2.3.4	Aerosol challenge	49
2.4	Results	51
2.4.1	Delayed-type hypersensitivity skin test	51
2.4.2	Lymphocyte proliferation assay	57
2.4.3	Aerosol challenge of vaccinated animals and comparison to lymphocyte proliferation results	71
2.5	Discussion	74
3.0	Investigation of the immune response to anthrax vaccination using a mouse model	78
3.1	Introduction	79
3.1.1	Murine models and anthrax investigations	79
3.1.2	Adjuvants	80
3.1.3	Recombinant protective antigen	82
3.1.4	Enzyme linked immunosorbent assay	83
3.1.5	Aim of the experiments	83
3.2	Method and materials	85
3.2.1	Experimental animals	85
3.2.2	Vaccination regimen	86
3.2.3	Lymphocyte proliferation assay	87
3.2.4	Determination of immunoglobulin sub-classes by ELISA	91

3.2.5	Passive transfer or reconstitution with immune components	93
3.2.6	<i>B. anthracis</i> spore challenge	93
3.3	Results	95
3.3.1	The response of NIH strain murine lymphocytes to native protective antigen	95
3.3.2	The immune response to native and recombinant <i>Bacillus anthracis</i> protective antigen, using a Balb/c murine model	101
3.3.2a	Day 30	101
3.3.2b	Day 35	103
3.3.2c	Day 41	106
3.3.2d	Day 79	109
3.3.3	Murine immune responses to recombinant <i>Bacillus anthracis</i> protective antigen	113
3.3.4	Reconstitution of SCID/Beige mice with immune cells and sera	119
3.3.5	Investigation of <i>B. anthracis</i> spore challenge in different mouse strains	122
3.3.5a	Challenge of SCID/Beige, CBA, Balb/c and C57BL6 mice with <i>B. anthracis</i> Ames spores	122
3.3.5b	Challenge of A/J and Balb/c animals with <i>B. anthracis</i> STI spores	124
3.3.6	Investigation of protection by two different vaccine preparations using A/J mice	126

3.3.7	Transfer of immune and non-immune sera and cells into naive animals	129
3.4	Discussion	131
4.0	Conclusions	134
	References	138



## List of Tables

Table 2.1	Cutaneous response 4 hours after intradermal injection of different amounts of Protective Antigen	52
Table 2.2	Cutaneous response 24 hours after intradermal injection of different amounts of Protective Antigen	53
Table 2.3	Cutaneous response 48 hours after intradermal injection of different amounts of Protective Antigen	55
Table 2.4	Cutaneous response 72 hours after intradermal injection of different amounts of Protective Antigen	56
Table 2.5	<i>In vitro</i> Lymphocyte proliferation response of guinea pig blood and spleen-derived lymphocytes exposed to native PA, mitogens and Ribi adjuvant	58
Table 2.6	Stimulation indices of guinea pig blood and spleen derived lymphocytes exposed to native PA, mitogens and Ribi adjuvant	61
Table 2.7	Summary of results for stimulation indices of blood and spleen derived guinea pig lymphocytes exposed to native PA, mitogens and Ribi adjuvant	63
Table 2.8	Statistical comparison of blood-derived lymphocytes from the different experimental groups	69
Table 2.9	Statistical comparison of spleen-derived lymphocytes from the different experimental groups	70
Table 2.10	Survivors 14 days after challenge with an aerosol of <i>B. anthracis</i> Ames spores	73
Table 3.1	Immunoglobulin isotypes detected at day 35 after vaccination	105
Table 3.2	Immunoglobulin isotypes detected at day 41 after vaccination	108

Table 3.3	Immunoglobulin isotypes detected at day 79 after vaccination	112
Table 3.4	Immunoglobulin isotypes of immune and non-immune C57BL6 sera used for reconstitution of SCID/Beige mice	120
Table 3.5	Protection of reconstituted SCID/Beige mice against challenge with <i>B. anthracis</i> Ames	121
Table 3.6	Survival of different mouse strains after <i>B. anthracis</i> spore challenge	123
Table 3.7	Susceptibility of two different mouse strains to <i>B. anthracis</i> STI spore challenge	125
Table 3.8	Survival of A/J mice after vaccination and challenge with two different levels of <i>B. anthracis</i>	128
Table 3.9	Protection of passively immunised A/J mice after challenge with <i>B. anthracis</i>	130

## List of Figures

Figure 1.1	World-wide distribution of anthrax in 1996/97	19
Figure 2.1	Concanavalin A induced proliferation by guinea pig lymphocytes	64
Figure 2.2	Phytohemagglutinin induced proliferation by guinea pig lymphocytes	66
Figure 2.3	Lymphocyte proliferation after stimulation with 5 µg/ml Ribi adjuvant	67
Figure 2.4	Lymphocyte proliferation after stimulation with 12.5 µg/ml native PA	68
Figure 3.1	Stimulation indices for different groups after stimulation by mitogens for 4, 5 or 7 days	97
Figure 3.2	PA-specific lymphocyte proliferation after 4 days stimulation	98
Figure 3.3	PA-specific lymphocyte proliferation after 5 days stimulation	99
Figure 3.4	PA-specific lymphocyte proliferation after 7 days stimulation	100
Figure 3.5	Lymphocyte proliferation responses to different concentrations (µg/ml) of native and recombinant PA at day 30	102
Figure 3.6	Lymphocyte proliferation responses to different concentrations (µg/ml) of native and recombinant PA at day 35	104
Figure 3.7	Lymphocyte proliferation responses to different concentrations (µg/ml) of native and recombinant PA at day 41	107
Figure 3.8	Lymphocyte proliferation responses to different concentrations (µg/ml) of native and recombinant PA at day 79	111
Figure 3.9	Lymphocyte proliferation responses to different concentrations (µg/ml) of native PA	115
Figure 3.10	Lymphocyte proliferation responses to different concentrations (µg/ml) of recombinant PA	116
Figure 3.11	Lymphocyte proliferation responses to different concentrations	

Figure 3.12	Immunoglobulin isotypes elicited by vaccination in different strains of mice	118
-------------	--	-----

Figure 3.13	Immunoglobulin isotypes induced by vaccine preparations in A/J mice	127
-------------	---	-----

## **CHAPTER 1.0**

### **Introduction**

## 1.0 Introduction

### 1.1 Classification

The family *Bacillaceae* comprises Gram-positive rod shaped bacteria that can produce endospores. The obligate anaerobic members of the family comprise the genus *Clostridium*. The genus *Bacillus* encompasses aerobes and facultative anaerobes and was first described by Cohn in 1872. This genus contains more than 60 species of aerobic or facultatively anaerobic Gram-positive bacilli. Most are saprophytic and found in soil, water, air and vegetation. In the presence of free oxygen, in conditions not conducive to growth and multiplication, the vegetative cells produce heat resistant endospores. Spores are the predominant form in the environment. *Bacillus* species have been implicated in serious infections associated with immunosuppression, traumatic wound and burns, surgical operating procedures, haemodialysis and food poisoning (Pearson, 1970; Tuazon *et al.*, 1979; de Boer & Diderichsen, 1991). The most well known human pathogen from within the genus is *Bacillus anthracis*, which gives rise to the disease, anthrax.

### 1.2 History

Anthrax has a well-documented history (Klemm & Klemm, 1959; Christie, 1987). Many consider the fifth and sixth plagues of Egypt described in chapter nine of Exodus to be systemic and cutaneous anthrax (Ebedes, 1981), and classical literature contains references to anthrax. For example, Virgil's description of a pestilence in wild and domestic animals in his third Georgic is thought to be anthrax (Dirckx, 1981).

In 1850, Rayer and Davaine established that anthrax was associated with growth of a filamentous non-motile bacillus in the blood of infected animals (Slater & Spitta, 1898). Observations by Brauell in 1857 extended this to the disease as it occurred in humans (Slater & Spitta, 1898), and the bacterial aetiology of the disease was clearly demonstrated by Robert Koch (1876). Three years later J.H. Bell, a physician in Bradford, concluded that *B. anthracis* was the causative organism of Woolsorters' disease (inhalational anthrax; Bell, 1880). A substantial accomplishment was the development, by Greenfield in England and Pasteur in France, of the active immunisation of animals against anthrax by attenuated bacterial vaccines (Greenfield, 1880; Pasteur *et al.*, 1881).

The pathogenesis of anthrax was extensively investigated during the first half of this century. Interest then fluctuated in response to influences such as the development of a successful live stock vaccine by Sterne (1937) and the arrival of penicillin therapy. An upsurge of interest in *B. anthracis* occurred during World War II when *B. anthracis* was considered as an agent for biological warfare (Rosebury & Kabat, 1947; WHO, 1970; Christopher *et al.*, 1997). Work on pathogenesis during the 1950's by teams at the Microbiological Research Establishment (MRE) at Porton Down in England and Camp Detrick in the USA led to an understanding of the toxigenic nature of the disease.

After the 1972 Biological and Toxin Weapons Convention was signed, widespread interest in *B. anthracis* again declined. After an outbreak of anthrax at Sverdlovsk in 1979, leading to 64 deaths following an accident at a military laboratory (Rich, 1980; Wade, 1980; Meselson *et al.*, 1994), alarm about the potential use of *B.*

*anthracis* as a biological weapon increased. It has been claimed that anthrax was used by parties involved in the insurrection in Rhodesia (Nass, 1992). However, suggestions have also been made that the disease may have been spread by flies (Anon, 1983; Turell & Knudson, 1987). Anthrax reappeared in the communications media in 1990 with the Iraqi invasion of Kuwait and their failure to leave before the United Nations Security Council deadline. UK and US military personnel sent to the Middle East as part of a multi-national UN force were vaccinated against a variety of diseases, including anthrax (Anon, 1991), because of concerns about potential use of biological agents (Spencer & Wilcox, 1993).

At present, at least 17 countries are thought to have offensive biological programs (Cole, 1996). *B. anthracis* is considered a credible threat (McGovern *et al.*, 1999; Inglesby *et al.*, 1999). The spores are easily produced, stored and delivered as an aerosol. Infection progresses rapidly, after aerosol exposure, with symptoms only evident shortly before death. Thus, treatment for anthrax acquired *via* the aerosol route is difficult (Friedlander *et al.*, 1993a; Inglesby *et al.*, 1999).

In recent years, anthrax has caused animal outbreaks in Australia, North America and Canada (Turner *et al.*, 1998; Elkin *et al.*, 1998). There have also been human and animal cases in parts of the Former Soviet Union (FSU) due to a breakdown in veterinary and public health networks (Woodhall, 1998; Hugh-Jones, 1998a, 1998b).



### 1.3 Morphology and cultivation

*B. anthracis* is a large Gram-positive, non-motile, spore-forming rod. The vegetative form is a square-ended rod, 1-1.5 µm wide by 4-10 µm long. In infected blood or tissues, the rods may be present in short chains, surrounded by a polypeptide capsule demonstrable with a microscope and polychrome methylene blue (M'Fadyean Reaction; M'Fadyean, 1903) or Indian ink (Turnbull *et al.*, 1993). In stained smears from colonies on nutrient agar plates, vegetative cells can be seen as long chains with no capsule, unless the medium contains serum (approximately 5 %) or bicarbonate (approximately 0.7 %) and is incubated under 5-20 % carbon dioxide (CO<sub>2</sub>; Carman *et al.*, 1985; Turnbull *et al.*, 1993).

The organism can be readily cultivated on nutrient agar and although it grows best aerobically, it can also multiply under anaerobic conditions. Nutritional requirements include thiamine and certain amino acids. Uracil, adenine, guanine and manganese all stimulate growth. Glucose, maltose, sucrose and trehalose are fermented by most isolates. Weak proteolytic and lecithinase activities can be demonstrated.

*B. anthracis* colonies are large, opaque, and white, with a rough surface and irregular edge when grown on blood agar. If viewed under a hand lens or dissecting microscope, colonies have a filamentous appearance. If cultured in the presence of 5 % CO<sub>2</sub>, *B. anthracis* forms a capsule and gives rise to smooth mucoid colonies.

Under unfavourable conditions, such as nutrient limitation, the vegetative organism sporulates. The process of sporulation occurs only in the presence of oxygen. The actual rate of sporulation is dependent on temperature and environmental factors

(e.g. pH, presence of certain cations etc.). The developing spore is visible, under phase-contrast microscopy or in stained smears, as an oval body centrally located within the bacillus, which does not distend the cell. The spores, unlike the vegetative organism, are exceedingly persistent (Böhm, 1990).

### 1.3.1 Persistence

Once formed, spores of *B. anthracis* can survive for long periods of time in different environments. They are resistant to heat, cold, ultraviolet irradiation, desiccation, high/low pH, chemical disinfectants and the metabolic products of other bacteria. Viable *B. anthracis* has been isolated from 200 year old bones uncovered at an archaeological site (De Vos, 1990), and anthrax spores prepared in 1888 by Pasteur were still viable 66 years later (Jacotot & Virat, 1954). Wilson and Russell (1964) reported the survival of anthrax spores in dry soil held for 60 years in a laboratory. A recent paper details the isolation of *B. anthracis* by cultural and molecular techniques, after storage inside a capillary tube hidden in a sugar cube for 80 years (Redmond *et al.*, 1998).

### 1.3.2 Virulence factors

*B. anthracis* possesses three major virulence factors, a poly- $\gamma$ -D glutamic acid capsule and two protein toxins. The role of the capsule has not been fully elucidated. However, it is known to be an essential virulence factor. There is evidence to show that capsule positive, toxin negative strains of *B. anthracis* can be

lethal in mice (Welkos *et al.*, 1993). It has also been reported that the capsule can inhibit host defence, *via* the prevention of phagocytosis of vegetative cells (Zwartouw & Smith, 1956). In conjunction with lethal and oedema toxin, the capsule allows virulent anthrax bacilli to grow practically unimpeded in the infected host (Keppie *et al.*, 1963). The capsule is a poor immunogen (Tomcsik & Ivanovics, 1938) and without toxin appears to be of little use in the formulation of a vaccine (Ivins & Welkos, 1988).

Early workers raised the idea of an anthrax toxin, but proving beyond doubt that a toxin was present was difficult (Eurich & Hewlett, 1930). The toxic nature of cell-free extracts from *B. anthracis* lesions have been known since 1904 (Watson *et al.*, 1947; Lincoln & Fish, 1970). Conclusive proof that death by anthrax was primarily due to the toxin release resulting from a bacteraemia came in 1954 (Smith & Keppie, 1954). It was found that if bacteraemia in guinea pigs was aborted by streptomycin treatment before a critical level, infection would be eliminated and animals would survive. If the critical level was exceeded before administration of antibiotics the animals died despite clearing the bacteraemia. Previously, it was thought that death was due to capillary blockage, hypoxia and nutrient depletion by the very high numbers of bacilli.

Demonstration that anthrax toxin has three components was provided by Stanley and Smith (1961). Some areas of the pathology remain unknown, including the relationship between toxin production, onset of bacteraemia and pathological features in the spleen and lymph nodes. Studies in primates have indicated that the toxin may depress electrical activity in the cerebral cortex, affecting the respiratory centre and contributing to anoxia, cardiac collapse, shock and sudden death (Vick *et*

*al.*, 1968).

Relatively recent investigations have suggested that *B. anthracis* may possess other virulence determinants, including S-Layer proteins (Fouet *et al.*, 1996, 1998) and extracellular proteases (Stepanov *et al.*, 1996a, 1996b; Stepanov & Leppla, 1996).

## **1.4 Toxin components**

The toxin complex contains 3 synergistic proteins: protective antigen (PA, 83 kDa), lethal factor (LF, 87 kDa) and oedema factor (EF, 89 kDa). The 3 components are produced during log phase growth of the organism. LF in combination with PA gives rise to Lethal Toxin (LT), and EF combines with PA to give Oedema Toxin (ET; Ezzell *et al.*, 1984). These two toxins are thought to be responsible for the clinical sequelae of anthrax infection by synergistic action (Pezard *et al.*, 1991).

### **1.4.1 Protective antigen**

Protective antigen is known to be important in the pathogenesis of anthrax toxin, hence its name (Watson *et al.*, 1947). It is a prerequisite in any formulation that provides immunity to the disease. Cell and animal culture investigations showed that LF and EF compete for available PA (Ezzell *et al.*, 1984). This suggested that PA may bind to a cell surface receptor and initiate an internalisation event, so that EF and LF can gain entry to the cytosol of the target cells (Leppla *et al.*, 1985).

Later research revealed that cell-bound PA is cleaved proteolytically from an 83 kDa species (PA83) at the target cell to give a biologically active 63 kDa (PA63) molecule for which both LF and EF compete and bind with high-affinity (Leppla *et al.*, 1988).

The crystal structure of PA shows that it is an elongated molecule of four domains composed of antiparallel  $\beta$ -sheets (Petosa *et al.*, 1997). Domain 1 (Residues 1-258) includes the major proteolytic fragment PA20 (Residues 1-167) and the putative binding site for LF and EF (Domain 1'; Residues 168-258). Protease cleavage of domain 1 occurs at amino acids 164-167 (Gordon *et al.*, 1995), present on an exposed loop (Petosa *et al.*, 1997). Removal of PA20 promotes formation of heptamers of PA63 (Petosa *et al.*, 1997; Milne *et al.*, 1994). Domain 2 (Residues 259-487) may be involved in heptamer formation by PA63 and membrane insertion (Petosa *et al.*, 1997; Milne *et al.*, 1994; Benson *et al.*, 1998). Domain 3 (Residues 488-595) may be involved in binding of EF and LF because of its close proximity to Domain 1' (Petosa *et al.*, 1997; Little *et al.*, 1996). Domain 4 (Residues 596-735) contains the cell-binding region of the molecule (Petosa *et al.*, 1997).

#### **1.4.2 Oedema toxin**

Suggestions that the oedema may be due to elevated levels of intracellular cyclic adenosine monophosphate (cAMP) led to determination of the mechanism of toxicity. The hypothesis was confirmed using a Chinese hamster ovary cell assay and EF (Leppla, 1982). EF is a calmodulin-dependent adenylate cyclase and therefore, only functional in eukaryotic cells (Leppla, 1982; Leppla *et al.*, 1985). It has a role

in a number of other processes that affect the outcome of infection. EF inhibits neutrophil functions (Alexeyev *et al.*, 1994), including phagocytosis (O'Brien *et al.*, 1985) and priming of the respiratory burst (Wright *et al.*, 1986). Hoover and co-workers (Hoover *et al.*, 1994) suggested that EF may have a role in the blocking of tumour necrosis factor-alpha (TNF- $\alpha$ ).

#### 1.4.3 Lethal toxin

Early work showed that LT induces pulmonary oedema and death in laboratory animals (Stanley & Smith, 1961; Beall *et al.*, 1962; Beall & Dalldorf, 1966). LT plays a bigger role in the pathogenesis of anthrax infection than ET. Inactivation of the EF gene in *B. anthracis* reduces virulence by 10-fold. However, inactivation of the LF gene reduces virulence more than 1000-fold (Pezard *et al.*, 1991).

LT is fast acting, causing death in Fischer 344 rats in as little as 38 minutes after intravenous injection (Haines *et al.*, 1965; Ezzell *et al.*, 1984). LT has been shown to lyse certain mouse macrophage cell lines on exposure to low concentrations of toxin in 90-120 minutes (Friedlander, 1986; Friedlander *et al.*, 1993b).

Studies by Hanna and others demonstrated that mice are rendered insensitive to anthrax LT by depleting their native macrophage populations using silica injections (Hanna *et al.*, 1993). Sensitivity could be restored by co-injection of lethal toxin and the toxin-sensitive monocyte/macrophage cell line, RAW 264. Further observations showed that sub-lytic levels of LT were capable of upregulating Interleukin-1 (IL-1) and TNF- $\alpha$  production. This was considered to correlate with the symptoms of

secondary shock observed in anthrax fatality (Hanna *et al.*, 1993). Experiments have demonstrated that elevation of superoxide anion correlates with cytolysis of macrophages exposed to lytic levels of LT (Hanna *et al.*, 1994). A hypothesis for the action of LT was developed. At low LT concentrations, superoxide anion induces cytokine production, IL-1 accumulates within the macrophage and TNF- $\alpha$  is released. As LT levels rise, increased levels of superoxide cause lysis of macrophages. Macrophage lysis leads to a sudden release of IL-1 $\beta$ , causing shock which kills the infected individual (Hanna, 1998).

Cell types not sensitive to LT have PA receptors and can respond to ET, suggesting an absence of an intracellular substrate for LF. Comparison of the LF amino acid structure with a protein database revealed a short region of similarity with a zinc-binding motif of zinc endopeptidases and a postulated inverted zinc-binding region, suggesting that LF functions as a metalloprotease (Klimpel *et al.*, 1994; Kochi *et al.*, 1994).

#### **1.4.4 Genetics and virulence**

The major virulence factors of capsule and toxin have been found to be plasmid-mediated. The genes *pag*, *lef*, and *cya* encoding the toxin components are located on a single 185kb plasmid, pX01 (Mikesell *et al.*, 1983), and the genes for capsule expression, polymerisation and export to the cell surface are located on a separate 90 kb plasmid, pX02 (Green *et al.*, 1985; Uchida *et al.*, 1985). Strains cured of one or both plasmids show avirulence or reduced virulence compared to strains with both plasmids (Ivins *et al.*, 1986).

## **1.5 Disease in animals**

World-wide, anthrax is an important disease in terms of economics and public health, because of its' ability to affect large numbers of livestock at the same time. The majority of animals contract gastrointestinal anthrax due to the ingestion of contaminated feed, grass, water or carcasses (Hugh-Jones & Hussaini, 1974; Redmond *et al.*, 1996; 1997). Lower numbers of cases of cutaneous anthrax may occur through the action of biting flies or contaminated wounds and abrasions. Inhalational anthrax may occur in rare cases.

Herbivores are usually highly susceptible to anthrax. Signs of disease may not be present until shortly before death. The animal becomes toxaemic, mild seizures can occur, followed by coma and death. Haemorrhage from the mouth and anus can occur in the final stages due to mucosal layer and cellular breakdown.

## **1.6 Disease in man**

Anthrax in man usually occurs after contact with dead infected animals or animal products. The disease can take several forms; cutaneous anthrax, which normally arises after the handling of infected bones, hides or products e.g. contaminated shaving brushes (Kobuch *et al.*, 1990; Turnbull *et al.*, 1990a; Fairbrother, 1962); intestinal anthrax, which occurs after ingestion of contaminated meat (Christie, 1987; Woodall, 1998), and inhalational anthrax, usually caused by an industrial



exposure to spores, for example, during processing of contaminated animal hides, wool and hair (Bell, 1880; Appleton, 1925; Albrink *et al.*, 1960). Anthrax meningitis can develop from any of the three classical forms.

### **1.6.1 Cutaneous anthrax**

The cutaneous form of the disease is acquired by contact through an existing or new abrasion. Cutaneous anthrax accounts for the greatest number of human cases of anthrax (95%; McGovern *et al.*, 1999). The primary lesion, a small pimple, appears after 2-3 days in the majority of cases, although this can range from 12 hours (Salmon, 1896) up to 19 days (Abdenour *et al.*, 1987).

The site of infection develops vesicles over the next 24 hours. The central papule ulcerates and dries to form the eschar, which enlarges and turns black covering the vesicles as they dry. The lesion is surrounded by oedematous tissue. After 5 or 6 days, the black eschar has firmly adhered to the underlying tissue.

In most cases the bacilli remain in the lesion. Inflammation of the regional lymph nodes (adenitis) is common. However, inflammation of the lymphatic vessels (lymphangitis) may indicate a secondary infection. Fever is usually mild or absent. Ten days after the initial appearance of the primary lesion, the eschar starts to resolve. This is a slow process, which takes 2-6 weeks. Scarring is usually minimal and treatment is only necessary when it affects functions, such as movement.

Septicaemia may occur as a complication. Chills, headache, anorexia and nausea

may appear before a rapid and fatal illness, with a rise in temperature to 38.9-40 °C followed by a rapid fall to below normal within hours. The patient becomes toxaemic and shocked. Dyspnoea, cyanosis and collapse precede death.

### **1.6.2 Intestinal anthrax**

In intestinal anthrax, lesions develop on the mucosal surface of the intestine following ingestion of *B. anthracis* spores in infected/contaminated meat. The incubation period is usually 3-7 days. The eschar occurs commonly on the wall of the terminal ileum or caecum; occasionally the oropharynx, stomach, duodenum or upper ileum may be affected. The lesion can appear gangrenous, the intestine and mesentery oedematous and the mesenteric lymph nodes enlarged. The earliest symptoms may range from mild gastrointestinal disturbance to nausea, abdominal pain and bloody diarrhoea (Turnbull *et al.*, 1993; Van den Bosch, 1996). The incubation period, like cutaneous anthrax, is 2-5 days. If an early diagnosis can be made, the disease can be treated (Section 1.6.5). Due to the difficulties in case identification, intestinal anthrax is more frequently fatal than cutaneous anthrax. Fatal cases show a sudden onset of malaise, with shock, collapse and death following within hours.

### **1.6.3 Inhalational anthrax**

This form of anthrax begins insidiously 2-5 days after inhalation of aerosolised *B. anthracis* spores. Spores are phagocytosed by lung macrophages and carried to the

regional lymph nodes in the thorax (Ross, 1957). No overt pulmonary infection occurs during inhalational anthrax and the lungs remain clear of vegetative bacilli (Ross, 1957). Spore germination and local multiplication occur within the macrophage. Vegetative cells eventually kill the macrophage and are released into the blood stream. As extracellular pathogens they can reach high levels (up to  $10^8$  bacteria per millilitre; Hanna, 1998). The first clinical signs are often mild fever, fatigue, malaise, myalgia, and a non-productive cough. The initial mild phase, lasting from two or more days, is followed by the sudden onset of acute illness. The patient shows acute dyspnoea and subsequently cyanosis and thus becomes moribund with an accelerated pulse and respiration. Haematemesis (vomiting blood) or haemoptysis (coughing up blood) can occur. Body temperature may be elevated or, if shock has occurred, may be lowered. The internal oedema gives rise to rales and signs of pleural effusion. The pulse becomes feeble and accelerated; dyspnoea, cyanosis, temperature changes and disorientation are followed by coma and death (Plotkin *et al.*, 1960). The disease is usually fatal (Legge, 1934), although successful treatment has been recorded (Plotkin *et al.*, 1960; see 1.6.5). It is possible that sub-clinical infection and consequent recovery can occur and be unrecognised (Christie, 1987).

#### **1.6.4 Anthrax meningitis**

Anthrax meningitis can arise as a complication of any form of the disease. It is difficult to differentiate clinically from other types of meningitis unless evidence of one of the other forms of disease is present. However, cerebral irritation may be present in some cases (Lalitha *et al.*, 1990). Clinical signs and appearance of blood

in the cerebrospinal fluid are followed by a rapid loss of consciousness and death (Knudson, 1986). The outlook in this form of the disease is poor.

### 1.6.5 Treatment

Antibiotic treatment usually leads to recovery of the individual or animal affected by cutaneous anthrax. Intestinal and inhalational anthrax are difficult to treat because symptoms are only evident shortly before death. Investigations have shown that penicillin can be used to treat natural and experimental *B. anthracis* infections (Barnes, 1947a; Gold, 1967). Most isolates of *B. anthracis* can be expected to be sensitive to penicillin (Lightfoot *et al.*, 1990; Odenaal *et al.*, 1991; Fellows, 1996), although penicillin-resistant isolates have been reported (Bradarić & Punda-Polić, 1992). The availability and low cost of penicillin mean that it remains the basis of treatment regimes, particularly in animals and in humans in the developing countries. *B. anthracis* is also sensitive to a wide range of broad-spectrum antibiotics (Knudson, 1986), including ciprofloxacin and doxycycline (Friedlander *et al.*, 1993a).

*B. anthracis* cannot be isolated from a cutaneous lesion 24 to 48 hours after start of therapy, although therapy is usually continued for 7 to 14 days. In cases of inhalational anthrax, spores have been shown to persist for many weeks in the lungs of monkeys on antibiotic therapy after infection *via* the aerosol route (Henderson *et al.*, 1956; Friedlander *et al.*, 1993a). Once therapy was withdrawn, the animals succumbed to the disease. It has been suggested that the chemical vaccine should be given simultaneously with antibiotic therapy (Henderson *et al.*, 1956; Lincoln *et al.*,

1965; Friedlander *et al.*, 1993a). The therapy should be continued for approximately six weeks to allow the development of vaccine-induced immunity (Friedlander *et al.*, 1993a). Treatment of the disease may include additional supportive therapy, such as administration of steroids, electrolyte stabilisation, tracheotomy and ventilatory support to ameliorate any shock symptoms that occur.

## **1.7 Epidemiology**

During the last 30-40 years there has been a reduction in the number of world-wide cases of animal and human anthrax. This is due to the wide scale use of the livestock vaccine and an increased awareness of disease control and hygiene methods. Thus the disease is sporadic in the majority of Europe. The major affected regions of Europe are those bordering the Mediterranean, including Greece, Italy, Spain, Turkey, and Yugoslavia. Notifications of human cases are very rare, but small outbreaks continue to appear in livestock in the United Kingdom (Turnbull, 1992; Turnbull *et al.*, 1996). Figure 1.1 shows the world-wide distribution of anthrax, including epidemic, endemic and anthrax free areas.

In Canada, incidence is ongoing in northern Alberta and the North West Territories (Elkin *et al.*, 1998). In the USA, it is confined to areas of North and South Dakota (Hugh-Jones, 1998c), Nebraska, Oklahoma, and Texas.

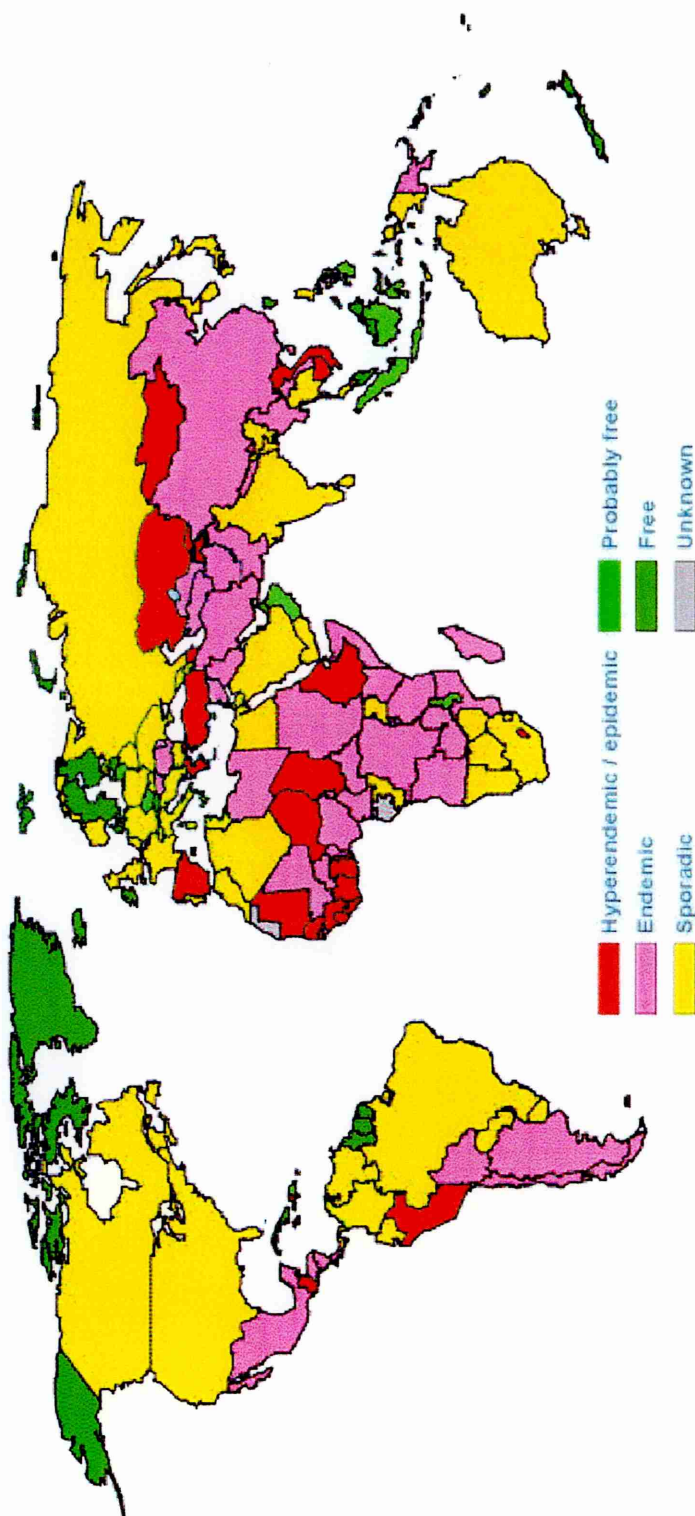
In Central and South America the true scale of the disease is uncertain. Under reporting and failure to investigate unexpected livestock deaths certainly occurs. Anthrax has been reported in Mexico, Guatemala and Honduras, although it is

absent in Belize and the Caribbean (except Haiti). In South America, Peru, Bolivia and Venezuela regularly suffer cases of the disease (Hugh-Jones, 1996; Turnbull *et al.*, 1998).

Africa remains severely affected with livestock and human anthrax, especially in West Africa. South Africa suffers from sporadic outbreaks (Turnbull *et al.*, 1998).

The traditional Middle Eastern and adjoining former USSR republics continue to suffer from anthrax (Hugh-Jones, 1998a). Outbreaks regularly occur in southern India, but anthrax is rare in the north of the country, although Nepal has endemic disease. The south-east Asian countries of Myanmar, Vietnam, and Cambodia are affected whilst Thailand, which used to be free of anthrax, are now experiencing endemic disease. Taiwan and Malaysia remain disease-free, and the disease in the Philippines and Indonesia is limited to single regions.

China is reported to suffer approximately 1000 cases per year, mostly in western areas of the country (Dongzheng, 1998).



**Figure 1.1** World-wide distribution of anthrax in 1996/7  
(from Hugh-Jones, 1999)

## **1.8 The Immune system and infection**

A function of an immune system is to protect the host against pathogenic microbes. Progression of an infectious disease in an individual involves numerous complex interactions between microbe and host. Key events during the process are entry of the microbe, invasion/colonisation of host tissues, interference with host defence mechanisms and functional impairment or injury. Some microbes produce disease by releasing toxins or inducing inflammation. Many factors determine the overall virulence and pathogenicity of microbes (Smith, 1995).

### **1.8.1 Immune response to infection**

The immune system is made up of a number of different cell types which together form an elaborate defence against infection. The system is broadly split into two sections, acquired and innate responses.

Acquired responses are specific for certain antigenic determinants (epitopes) and are amplified by contact with those epitopes. An epitope is the small site on the antigen to which the antibody attaches. The cells involved are B-lymphocytes that develop into antibody-secreting cells, and T lymphocytes which form cells responsible for cell-mediated immunity. Any one B or T cell, or their clonal descendants, recognise a single, specific epitope.

The innate immune response is brought about by macrophages, dendritic cells, polymorphonuclear leukocytes (PMNL), natural killer (NK) cells, the complement



system, and cytokines. These all act non-specifically, although their response can be enhanced or suppressed during infection. The two arms of the immune response act in a collaborative and highly orchestrated fashion, although it is possible for only one aspect to be responsible for the elimination of a particular infection.

### **1.8.2 B lymphocytes and antibodies**

Antibodies (immunoglobulins) are produced by plasma cells, which have formed from B-lymphocytes after activation by an antigen. Prior to this activation, each cell has multiple copies of a specific receptor already inserted into its plasma membrane. This receptor is identical to the antibody that the cell will eventually produce. Exposure to the antigen causes the B-lymphocytes to divide and differentiate into plasma cells and memory cells. The plasma cells are stimulated to produce and secrete antibody specific for the activating antigen. Memory cells have the same specificity but do not secrete antibody. Exposure to the same antigen for a second time causes these cells to develop into antibody-secreting plasma cells, which produce an enhanced immune response to prevent reinfection.

In man, there are five types of immunoglobulin: IgA, IgG, IgD, IgE, and IgM. Immunoglobulin (Ig) can bind directly to the antigen, or to cells that are expressing antigenic proteins/peptides after infection. Antigens or infected cells that have been recognised by antibody, may then become targets for the complement system (Roitt, 1991), or for phagocytic cells which carry receptors for the antibody in their plasma membrane. The killing of infected cells by phagocytes in this way is known as antibody-dependent cellular cytotoxicity (ADCC).

### **1.8.3 The complement system**

The complement cascade comprises a nine-component enzyme system that is initiated by IgG and IgM. Once the first component is activated, this activates the next by proteolytic cleavage and so on, with progressive amplification. Cleavage of each component produces two parts, one of which binds to the antigen-antibody complex, and a diffusible part that forms a chemical gradient to attract cells of the innate immune response. At the final stage, a pore structure is inserted into the membrane of the antigen or infected cell. Once a sufficient number have been inserted, this will bring about an ionic imbalance and eventual lysis of the antigen or cell.

### **1.8.4 T lymphocytes and cell-mediated immunity**

The cell-mediated component of the adaptive arm of the immune system consists of T lymphocytes. Each cell has many copies of a unique receptor on its surface, the T cell receptor (TCR), that will recognise the epitope. Unlike B cells, T cells do not secrete their receptor but react only with epitopes expressed on the surface of other cells. This expression is brought about by intracellular proteolytic processing of the foreign antigen. During processing, the peptides are complexed with host proteins called major histocompatibility complex class I or II (MHC I or MHC II). The complex is transported to the surface of the cell where the peptide is displayed (antigen presentation). The peptide presented on the surface of an infected cell is

recognised by the TCR. Activation of the T cell then follows and causes clonal expansion and differentiation of the cell into a functional T cell. These then fall into two categories; CD8<sup>+</sup> T cells recognise peptide associated with MHC I, whilst CD4<sup>+</sup> T cells recognise peptide associated with MHC II. Recognition of the MHC is in addition to recognition by the TCR. All cells of the body express MHC I molecules and are, therefore, under surveillance by CD8<sup>+</sup> T cells. Under normal conditions, only immune cells express MHC II molecules, although other cells can be induced to express MHC II molecules during infection of the host. Therefore, T cells only recognise a foreign peptide once it has been complexed with an MHC molecule. An exception to this is a population of T-cells, termed the  $\gamma\delta$  cells, which can respond to a foreign antigen directly. They appear to play a role early in defence against infection. Experiments using gene deletion mice have shown a role for  $\gamma\delta$  cells in protection against experimental tuberculosis (Ladel *et al.*, 1995).  $\gamma\delta$  T-cells show a rapid response to infection and may be a link between the innate and acquired immune system (Boismenu & Havran, 1997).

Fully activated T cells can have either an effector or a regulatory function. At times, these functions can appear to be contradictory. This may be brought about by different sub-populations, or it may depend upon the activating signal that the cell receives. The main T cell functions are as follows:

- a) *Cytotoxicity*, in which cells that are expressing processed foreign antigens are destroyed. This is mostly carried out by CD8<sup>+</sup> T cells.
- b) *Delayed-type hypersensitivity*, is the result of the secretion of cytokines, such as  $\gamma$ -interferon and interleukin-2, which are soluble proteins that have a wide number of effects. Other cytokines affect macrophages and PMNL, recruiting them to the site of infection and altering blood vessel permeability in that area. This causes

inflammation, a swelling and reddening at the site due to the influx of immune cells and fluid containing antibodies. Although helpful in moderation, in excess it can cause tissue damage.

c) *Help*, assists B and T lymphocytes to mount the correct immune response. Therefore, the whole immune response depends on T cell help. This is brought about entirely by CD4<sup>+</sup> T cells.

d) *Suppression*, is not well understood. It controls the immune responsiveness of other cells in opposition to CD4<sup>+</sup> T cells. It is thought to involve CD8<sup>+</sup> T cells as well as, to a lesser extent, CD4<sup>+</sup> T cells.

Once activated naive CD4<sup>+</sup> Th cells differentiate into two subpopulations, Th<sub>1</sub> and Th<sub>2</sub>. The cell subsets are based on the characteristic profile of cytokines they produce, and the role they play in host defence. Th<sub>1</sub> cells coordinate cell-mediated immunity and produce IFN- $\gamma$ , IL-2 and TNF- $\beta$ . Secretion of IFN- $\gamma$  leads to macrophage activation, induction of B lymphocytes to produce IgG1 and IgG3 and increased expression of MHC class I and II on many cells. IL-2 stimulates proliferation of B cells, activated T cells and NK cells. TNF- $\beta$  plays a role in macrophage activation leading to enhanced killing of intracellular bacteria.

Humoral immunity is coordinated by Th<sub>2</sub> cells, which secrete IL-4, IL-5 and IL-10. The role of Th<sub>2</sub> is to provide cytokines required for immunity to extracellular pathogens. IL-4 has a number of roles, including stimulation of B cell proliferation, stimulation of mast cell growth, elevation of MHC class II on B cells and switching to IgG and IgE production. IL-5 stimulates class switching to IgA and promotes growth of eosinophils. IL-10 increases production of mast cells and increases expression of MHC class II on B cells.

The type of response that is elicited is vital to host defence. In general, intracellular infections elicit a Th<sub>1</sub> response and cell-mediated immunity, whereas extracellular infection elicits Th<sub>2</sub> cells and antibody-mediated reactions. An inappropriate response may lead to persistence of the pathogen with serious consequences. Studies of *Mycobacterium leprae*, an intracellular bacterium causing leprosy, have underlined the importance of appropriate responses. Some individuals are predisposed to an effective Th<sub>1</sub>-mediated response and clear the infection. Other people mount a Th<sub>2</sub>-mediated response and are unable to clear the infection.

#### **1.8.5 Other cells of the immune response**

Macrophages can engulf, or phagocytose, foreign antigens. This is a non-specific response, although specificity occurs when the antigen or infected cell has been recognised by antibody. In this case, the macrophage has receptors in its plasma membrane that will recognise the tail (Fc region) of the antibody. Recognition then brings about phagocytosis of the antibody-coated (opsonised) antigen/cell. Macrophages are found in blood as monocytes and can differentiate from blood monocytes to macrophages to enter organs and tissues, whilst the PMNL, another family of phagocytes, are found in the peripheral blood.

NK cells recognise virally infected cells. They arise early in infection, within 2 days, and bring about non-specific lysis of the cell. This cytotoxic activity is not dependent on antibody.

### 1.8.6 Vaccines

Vaccines exploit the phenomenon of immunological memory. Primary exposure to vaccine antigens elicits a pool of memory T and B-lymphocytes. Subsequent exposure to the same antigens, upon contact with the pathogen, leads to activation of the lymphocytes and clearance before disease occurs.

Conventional vaccine design has changed little in the last century. Two basic methods are used: inactivation or attenuation of the pathogen. Inactivation can be carried out using chemical fixatives, denaturation by heat or exposure to radiation. An example of an inactivated vaccine is the whole cell typhoid vaccine. This is produced using heat killed, phenol preserved *Salmonella typhi* cells. Inactivated vaccines may be able to induce an antibody response but may not efficiently induce cellular memory. Antigen persists for a relatively short period of time leading to short term immunity. Thus, regular booster vaccinations are required to maintain levels of serum antibody. Inactivated vaccines are relatively easy to store. However, they carry the risk of under- or over-inactivation. Inadequate treatment could leave viable pathogens or toxins in the vaccine preparation. Over inactivation may lead to excessive denaturation and change of the three-dimensional structure of the antigen, leading to antibody that fails to react with native antigen of the pathogen. Further, these formulations do not lend themselves easily to mucosal dosing to induce appropriate mucosal immunity.

Attenuated vaccines are especially adapted strains of live microorganisms that retain infectivity but are non-pathogenic. Attenuation relies on naturally occurring

variability of virulence within a population. In practice, attenuation is a time-consuming trial and error process. It is achieved by sub-culturing pathogens in unnatural hosts or *in vitro* to select strains with reduced virulence. Attenuated pathogens retain their ability to infect cells and elicit T lymphocyte and antibody responses. Live vaccines persist for longer than inactivated vaccines and generally lead to long-lasting immunity, without repeat vaccination. The main disadvantages associated with an attenuated vaccine are the risk of reversion to virulence and requirement for cold storage, which can make use difficult in some countries.

Recombinant DNA technology has been used to produce genetically attenuated vaccines. Vaccines attenuated by recombinant techniques have a number of advantages. The organism can be attenuated by deletion at two different sites. Thus, the probability of reversion to virulence is greatly reduced. Attenuated pathogens can be used as vectors for foreign genes encoding the antigens of other pathogens. After immunisation, the recombinant organism expresses its own antigens and the foreign genes that provide antigen for acquired immunity.

Recombinant purified vaccine antigens may be used to construct a sub-unit vaccine. Recombinant expressed proteins have the same immunological properties as inactivated conventional vaccines. Use of recombinant antigen means that the final vaccine formulation can be composed of vaccine antigen and adjuvant, without contaminating media components. Sub-unit vaccine composed of purified polypeptides have been used for Hepatitis B and Influenza virus

### 1.8.7 Adjuvants

Inactivated vaccines can be made more immunogenic using adjuvant compounds. When adjuvants are added to vaccines they enhance the immunogenicity of an antigen in stimulating a humoral response. In some cases, adjuvants may elicit a cell-mediated response to antigen. The only adjuvants currently licensed for human use are aluminium salts. Use of aluminium-based adjuvants started in 1926 (Glenny, 1926). Consequently there is accumulated data available for safety purposes.

During vaccine development, experimental adjuvants, such as water-in-oil, bacterial-derived and copolymers may be used to increase the immune responses and determine which antigens are important in protection.

### 1.8.8 Animal models

An increasing number of investigations utilise *in vitro* techniques to look at different aspects of the immune system, including phagocytosis, signal transduction and cytotoxic activity. However, animal models play an important role in understanding how microorganisms interact with the host. Information on pathogenicity and virulence mechanisms of a number of bacterial and viral diseases, including methods of immune evasion are now available. When studying interactions of microorganisms and their hosts, it is important to use models that mirror the disease under investigation (Smith, 1964). A variety of animal models exist to study infectious disease, caused by a number of pathogens (Clarke & Bavoil, 1994).



The best experimental models for human infection are usually primate-based due to the close relationship to humans. However, ethical, financial and conservation considerations restrict their use. Logistical and financial reasons mean that the majority of models exploit small laboratory animals, such as rats and mice. The complexity of the immune system means *in vivo* experiments are indispensable from an experimental and pharmaceutical licence viewpoint.

Murine models are important in terms of investigation of pathology, treatment and vaccine development (Williamson *et al.*, 1997; Roberts *et al.*, 1998; Russell *et al.*, 1998). Naturally occurring and artificially induced immunodeficient mouse strains have been used to study a number of aspects of immunity (Viney, 1994) and pathogenesis of infectious disease (Kaufmann & Ladel, 1994). A recent tool for vaccine assessment is the Severe Combined Immunodeficient (SCID) mouse. The SCID mouse has no functioning immune system and can be reconstituted with components of the human immune system, including lymphocytes from peripheral blood, thymus and spleen (McBride *et al.*, 1993). A variant of the SCID model has been used to study protection against bacterial infection during vaccine development (Green *et al.*, 1999).

#### **1.8.9 Animal models and anthrax**

A number of different species have been used to investigate the efficacy of anthrax vaccines, including mice (Welkos & Friedlander, 1988), guinea pigs (Sterne, 1937; McBride *et al.*, 1998), primates (Darlow *et al.*, 1956; Ivins *et al.*, 1998), rats (Mahlandt *et al.*, 1966), rabbits (Gladstone, 1946; Pitt *et al.*, 1998) and sheep

(Pasteur *et al.*, 1881; Sterne, 1937). The most appropriate model must be chosen to model human disease. All models have limitations. Investigation of the susceptibility of different species to infection with anthrax spores or the action of the toxin has shown wide variations (Lincoln *et al.*, 1967). The non-human primate has been considered the best model for inhalational anthrax. The problems of primate use in terms of availability, finance, conservation and logistics, dictate that wide ranging studies cannot be carried out. The alternative is a small laboratory animal based model. The choice of species for use to model infection and determine immune correlates of protection is difficult. The use of guinea pigs and mice for anthrax investigations will be discussed in later chapters.

## **1.9 Anthrax vaccination**

### **1.9.1 History**

Anthrax vaccines have been available for over a century. William Greenfield, Professor Superintendent of the Brown Animal Sanatory Institution (1871-1881), prepared an effective vaccine against anthrax in 1880 (Greenfield, 1880; Wilson, 1979; Tigertt, 1980). He used growth at high temperature (42-43 °C) to attenuate *B. anthracis* (Greenfield, 1880). However, his achievement was not recognised at the time because of financial and political considerations. Discovery that the virulence of *B. anthracis* could be reduced was pivotal to development of an effective vaccine.

The Pasteur vaccine (Pasteur *et al.*, 1881) was a two-stage vaccine using *B.*

*anthracis* prepared by two different protocols (Type I and II; Ezzell *et al.*, 1985). Primary inoculation used a sub-culture of *B. anthracis* that had been incubated at 42-43°C for 15-20 days (Type I vaccine). Type I vaccine had lost the ability to sporulate and was only pathogenic for mice or young guinea pigs (Ezzell *et al.*, 1985; Hambelton *et al.*, 1984). Type II cultures were taken after 10-12 days of growth at 42-43°C. These were consequently less attenuated than Type I cultures. The vaccine was used experimentally at Pouilly-le-Fort in May 1881 with success. This led to widespread use of live vaccines in cattle and sheep in Europe and South America with significant reduction in animal mortality from anthrax.

The mechanism of attenuation, by sub-culture at elevated temperature was not understood until 1983. Mikesell and others produced evidence that toxin production in *B. anthracis* was plasmid-mediated. When virulent strains were passaged at 42.5°C, there was a loss of detectable LF and EF. Transformation of heat cured strains with parental plasmid DNA resulted in restoration of toxin production (Mikesell *et al.*, 1983).

Pasteur's duplex vaccine was widely used from the late 1800's until 1935 (Turnbull, 1991). Although effective and valuable, heat attenuated vaccines suffered from variations in virulence, and were superseded by attenuated live spore vaccines.

### **1.9.2 Attenuated live spore vaccine**

The most significant event in the control of anthrax was the development of a live, attenuated spore vaccine by Sterne (1937; 1939). This vaccine and its derivatives

have proved effective as veterinary vaccines and helped to reduce economic loss due to anthrax. The Sterne vaccine uses a toxigenic, non-capsulating *B. anthracis* strain, 34F2 (Sterne, 1937). The strain was a rough avirulent culture derived from the sub-culture of a bovine isolate on 50% horse serum nutrient agar, followed by incubation under an atmosphere of 30% CO<sub>2</sub> for 24 hours. The final formulation consisted of 10<sup>6</sup> spores/ml suspended in 0.5% saponin in 50% glycerine-saline (Sterne, 1939).

The main limitation of live spore vaccine is safety. Attenuated strains retain a low level of virulence in some animal species (Sterne, 1939; Cartwright *et al.*, 1987). It is because of the safety concerns that live spore vaccines are generally considered unsuitable for human use in western countries. However, live spore vaccines have been developed and used in China (Dong, 1990) and the FSU and its Republics (Rimmington, 1994; Shlyakhov & Rubenstein, 1994).

### 1.9.3 Chemical vaccine

The possibility of using non-living anthrax vaccines was mentioned by Toussaint (Sterne, 1959). Bail reported observations concerning the existence of an immunogenic substance in the sterile oedema fluid from dying animals (Bail, 1904a,b). Studies over the next 50 years culminated in an understanding of the toxigenic nature of the disease and recognition of the PA component.

Gladstone isolated a protective antigen in cultures of *B. anthracis* grown *in vitro* in a complex medium consisting of blood plasma and serum from various animal

species (Gladstone, 1946). The properties of culture filtrates were similar to those of oedema fluid in terms of immunogenicity. Rabbits, monkeys, sheep and guinea pigs were protected, but not mice (Gladstone, 1948). The quantity of PA produced was small in relation to the amount of serum protein present, making purification difficult.

Boor and Tresselt produced and tested another *in vitro* produced anthrax vaccine (Boor, 1955; Boor & Tresselt, 1955a). The *B. anthracis* strain used was of intermediate virulence and grown in static culture. The medium consisted of serum albumin of a species homologous or genetically related to that to be immunised, plus modified yeast extract, phosphate and bicarbonate. Bacteria were removed by centrifugation and filtration. The antigen was concentrated by precipitation with ammonium sulphate or alcohol, followed by freeze-drying. Preparations were effective for immunising guinea pigs, sheep, non-human primates, and rabbits against  $10^3$ - $10^4$  lethal doses of virulent anthrax spores (Tresselt & Boor, 1955; Boor & Tresselt, 1955b). The drawback was the inclusion of large amounts of serum proteins in comparison with the amount of PA in the culture. This led to difficulties with purification and characterisation.

Workers in the UK (Belton & Strange 1954) and USA (Wright *et al.*, 1954) produced PA using non-proteinaceous media. PA could be separated from other toxin components and immobilised by alum precipitation or adsorbed with aluminium hydroxide. This led to production of chemical vaccines. The UK vaccine is an alum-precipitated cell-free culture filtrate of *B. anthracis* strain 34F2. The US vaccine is an aluminium hydroxide-adsorbed cell-free culture filtrate of a non-encapsulating, non-proteolytic derivative of a *B. anthracis* bovine isolate, V770

(Puziss & Wright, 1963). Such vaccines have been shown to produce some protective activity in experimental animals (Darlow *et al.*, 1956; Turnbull *et al.*, 1986, 1988; Ivins *et al.*, 1998) and may be effective in humans (Brachman *et al.*, 1962). Other non-living chemical vaccines have been reported in the FSU (Aleksandrov *et al.*, 1960) and China (Dong, 1990), however, their use appears to be limited.

#### **1.9.4 Efficacy problems**

Brachman and co-workers were able to compare anthrax case rates at the time the US vaccine was administered to at-risk workers (Brachman *et al.*, 1962). The data showed a 92.5 % reduction in the number of anthrax cases. This retrospective study is the only study showing data on the efficacy of the vaccine in humans. Investigations have shown that the vaccine confers good protection against Vollum and Vollum-derived strains of *B. anthracis* in Rhesus monkeys (Darlow *et al.*, 1956) and guinea pigs (Broster & Hibbs, 1990). A number of studies have shown that protection is less effective in experimental animals challenged with spores from other strains of *B. anthracis* (Auerbach & Wright, 1955; Ward *et al.*, 1965; Little & Knudson, 1986; Broster & Hibbs, 1990; Ivins *et al.*, 1994; Jones *et al.*, 1996a; Fellows *et al.*, 1998).

#### **1.9.5 Immunological basis of protection**

Protection against anthrax in a susceptible host is nearly entirely dependent on the

host's immune response to PA (Little *et al.*, 1997). Immune responses to LF and EF may help in protection against infection (Ivins & Welkos, 1988; Turnbull *et al.*, 1988). To date, no other antigens have been identified that contribute to protection so far. However, it can be seen that immunological responses to other virulence factors (e.g. S-layer proteins) may play a role in protection against infection.

A large number of studies have shown that protection against anthrax infection cannot be correlated to antibody titre against PA (Ivins *et al.*, 1986, 1990, 1992, 1994, 1998; Turnbull *et al.*, 1986, 1988, 1990b; Ivins & Welkos, 1988; McBride *et al.*, 1998). Efficacy of licensed and experimental vaccines and correlation with protection is dependent upon which animal model is used (Section 1.8.9). This could be due to differences in host susceptibility or the relative importance of various mechanisms of immunity to anthrax among animal species. Primates can be protected against inhalational anthrax using the current licensed human chemical vaccines (Darlow *et al.*, 1956; Pitt *et al.*, 1996; Ivins *et al.*, 1995; 1998), but an *in vitro* correlate has yet to be found (Ivins *et al.*, 1998). It has been reported that a model using rabbits shows a correlation between IgG and toxin neutralising antibody levels and protection against an inhalational challenge (Pitt *et al.*, 1998). Guinea pigs are protected by immunisation, with Sterne live spore vaccine or highly adjuvanted PA experimental vaccines, to a greater extent than with currently licensed human chemical vaccine (Little & Knudson, 1986; Ivins *et al.*, 1992, 1995; Turnbull *et al.*, 1990b; Jones *et al.*, 1996a), although anti-PA titres were lower for live spore vaccinated animals (Ivins & Welkos, 1988; Little & Knudson, 1986; Ivins *et al.*, 1992; Welkos & Friedlander, 1988; Welkos *et al.*, 1990). This may indicate that the current assays for PA antibody do not adequately detect antibody to protective epitopes, or that other immune mechanisms or components are involved

in protection (Broster and Hibbs, 1990; Ivins & Welkos, 1988; Ivins *et al.*, 1992, 1996, 1998; Little & Knudson, 1986; Turnbull 1991; Turnbull *et al.*, 1990b). Investigations have shown that PA-deficient strains can provide some protection, which constitutes additional evidence suggesting that immune mechanisms other than humoral immunity are involved (Pezard *et al.*, 1995).

Observations have been made that the addition of non-specific cellular material such as killed *Bordetella pertussis*, *Corynebacterium ovis*, Freund's complete adjuvant and Ribi adjuvant preparations to either the human vaccines or purified PA enhanced protection (Ivins *et al.*, 1992; Turnbull *et al.*, 1988, 1990b).

Shlyakhov and co-workers, in the FSU and Israel, have investigated a preparation called 'Anthraxin' (Aleksandrov *et al.*, 1959; Shlyakhov & Rubenstein, 1996). Anthraxin was originally derived from the oedematous fluid of research animals that had been infected with either the STI vaccine strain or the capsulated Zenkowsky strain of *B. anthracis* (Pfisterer, 1990; Shlyakhov *et al.*, 1997). It was an autoclaved liquid composed of a heat-stable polysaccharide-protein-nucleic acid complex and does not contain anthrax toxin or capsule material, preservative or antimicrobials (Shlyakhov *et al.*, 1997).

Anthraxin has been used widely to provide retrospective and early diagnosis of anthrax infection in the FSU for the last 40 years (Shlyakhov & Rubenstein, 1996; Shlyakhov *et al.*, 1973; Shlyakhov, 1996), although to date there has only been one study in a western country, Switzerland (Pfisterer, 1990). Other investigations have reported that anthraxin can be used to monitor immunity to anthrax following vaccination (Shlyakhov, 1971; Shlyakhov, 1996; Shlyakhov & Rubenstein, 1996;



Shlyakhov *et al.*, 1997; Shlyakhov, 1998; Aleksandrov *et al.*, 1959). After 18-24 hours, intradermal injection elicits local erythema and skin induration in acute and recovered anthrax patients, and live spore vaccinated animals and humans (Shlyakhov *et al.*, 1994; Shlyakhov, 1996). The cutaneous response can still be seen 48 hours later. Histological and immunological studies confirmed the reaction was due to a delayed-type hypersensitivity reaction (Shlyakhov & Shroit, 1964).

Efficacy of the chemical vaccine containing an aluminium adjuvant points to a role for humoral immunity (Brachman *et al.*, 1962). Individuals showing a delayed type hypersensitivity reaction to *B. anthracis* antigens after immunisation suggests a role for Cell-mediated immunity (Shlyakhov, 1996). It may be that neither cellular nor humoral immunity predominate in protection against *B. anthracis* infection.

#### **1.10 Aim of the investigation**

The aim of this investigation is to employ immunological techniques, experimental vaccines and animal models to shed more light on the mechanism of protection against *B. anthracis* infections. It is hoped that the results will give rise to information on immune correlates of protection. This would allow a method of determining an individuals' immunological status in terms of protection against *B. anthracis* infection. This information will provide much needed data to public health authorities/bodies and other concerned parties, and lays the basis for the development of a second-generation anthrax vaccine.

## 1.11 Organisation of the investigation

The investigation of the cellular immune response to anthrax vaccination in a guinea pig model and preliminary investigation of an NIH mouse model took place at the Centre for Applied Microbiology and Research, Porton Down, UK.

Further investigation of the murine immune response to anthrax vaccination took place at Chemical and Biological Defence, DERA Porton Down, UK. Challenge experiments with *B. anthracis* spores took place at the Centre for Applied Microbiology and Research, Porton Down, UK.

## CHAPTER 2.0

Investigation of the cellular immune response  
to anthrax vaccination using a guinea pig model

## **2.0 Investigation of the cellular immune response to anthrax vaccination using a guinea pig model**

### **2.1 Introduction**

#### **2.1.1 Anthrax investigations and guinea pig models**

Guinea pigs have been used for a large number of studies over a number of decades. They were used for initial efficacy investigations on the live spore vaccine (Sterne, 1937), as well as elucidation of the toxigenic nature of anthrax by workers in the UK and USA. Guinea pigs were used for biochemical, toxicological and bacteriological investigations (Smith & Keppie, 1954; Lincoln *et al.*, 1967).

In the 19th century, guinea pigs were used in early inhalational anthrax investigations (Barnes, 1947b). Workers developed the guinea pig model of inhalational anthrax (Barnes, 1947b; Druett *et al.*, 1953) as a tool to study the pathogenesis of the inhalational form of the disease (Ross, 1957). Since the late 1950's, this model has been used to investigate the efficacy of different vaccines and treatments against inhalational anthrax (Vančurík, 1966; Ivins *et al.*, 1986; Broster & Hibbs, 1990; Jones *et al.*, 1996ab; McBride *et al.*, 1998).

Guinea pigs have been characterised as susceptible to spore infection, but resistant to toxin (Ezzell *et al.*, 1984; Little & Knudson, 1986). Investigations of the efficacy of licensed human vaccines have reported partial protection to cutaneous infection (Ivins *et al.*, 1994). However, only minimal protection was seen against inhalational infection (Jones *et al.*, 1996a; Ivins *et al.*, 1995). No *in vitro* correlate of immunity

has been found in the guinea pig model (Ivins *et al.*, 1986; Turnbull *et al.*, 1986, 1988, 1990b; Ivins & Welkos, 1988; McBride *et al.*, 1998).

### 2.1.2 Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) skin tests have been used to study immunity to a number of microbial agents and their components for at least two centuries. Edward Jenner described delayed papular erythematous skin lesions in persons revaccinated against smallpox as a reaction of immunity in 1798. Skin tests for DTH against *Mycobacterium tuberculosis* are still used before vaccination to determine an individuals' sensitivity to tuberculin proteins. The anthrax skin test (Section 1.9.5) from the former USSR is based on a tuberculin-type skin test to measure a DTH reaction (Shlyakhov *et al.*, 1997).

DTH is a form of cell mediated immunity in which the effector cell is the sensitised T-lymphocyte. The classic model of DTH is the delayed response (up to 48 hours) of a sensitised guinea pig to antigen applied by 'skin painting' or intradermal injection. The DTH response is usually measured as erythema and induration responses in the ear pinna or footpad, due to the ingress of sensitised T-cells causing an inflammatory cascade.

DTH reactions consist of three phases: recognition/activation, inflammation, and resolution. During recognition/activation, T<sub>H</sub> CD4<sup>+</sup> cells (and sometimes CD8<sup>+</sup> T cells) recognise antigen presented on the surface of antigen-presenting cells and respond by producing cytokines. Recognition/activation occurs in the peripheral

tissue where memory and effector T cells are recruited. The inflammation phase occurs when vascular endothelial cells, activated by cytokines or contact with activated T cells, recruit circulating leukocytes into tissues at the site of antigen challenge. The resolution phase occurs when macrophages activated by cytokines act to eliminate the antigen. This phase may lead to tissue injury.

### 2.1.3 Lymphocyte proliferation assay

Cells of the immune system activate, proliferate and differentiate into effector cells upon challenge by invading pathogens. The cellular response can vary depending upon a number of variables, including nature of the pathogen (i.e. intra or extracellular), route of challenge and dose.

*In vitro* assays can be performed to provide information about lymphocytes, from basic proliferation by a mixture of cells to use of isolated and highly purified lymphocyte subsets. After antigenic stimulation, lymphocytes expand quickly by repetitive mitotic cycles. Each cycle of cellular division requires progression of the cell cycle and DNA synthesis. Expansion of a population of cells correlates with the rate and amount of DNA synthesis. This has led to measurement of DNA synthesis as a measure of proliferation. The labelled nucleoside [methyl-<sup>3</sup>H]thymidine competes with endogenous thymidine for incorporation into newly synthesised DNA. The quantity of radiolabelled DNA can be determined by harvesting cells onto a support material, such as glass-fibre filter mats. The support traps genomic DNA, but allows oligonucleotides or single bases to pass through. The support can be counted on a  $\beta$ -radiation counter using liquid scintillation counting. The counts for

stimulated and unstimulated cells can be compared.

## **2.2 Aim of the experiments**

The primary aim of the DTH experiments was to determine if purified PA can elicit a DTH response in previously vaccinated guinea pigs. The secondary aim was to determine if the response varied due to different vaccine formulations.

The aim of the lymphocyte proliferation study was to investigate if vaccination, using different vaccination regimen, led to *in vitro* proliferation of lymphocytes upon second exposure to antigens from the vaccines, indicating establishment of T-cell memory, a desirable objective. A further aim was to challenge cohorts vaccinated by the selected vaccination regimen and determine if there was any correlation with protection.

## **2.3 Method and materials**

### **2.3.1 Experimental animals**

Experimental guinea pigs were housed in groups of one or two. All received food and water *ad libidum*. The guinea pig used were a barrier-reared Dunkin-Hartley strain (Harlan UK Ltd, Oxfordshire, UK). They were approximately 350 g at the time of the first immunisation. All investigations involving animals adhered to the requirements of the Animal (Scientific Procedures) Act 1986.

### **2.3.2 Delayed-type hypersensitivity skin test**

A total of ten guinea pigs were vaccinated by intramuscular injection at 0, 3 and 6 weeks. Four animals were vaccinated with one-tenth of a human dose of vaccine (0.05 ml UK human vaccine and 0.05 ml saline), four animals were vaccinated with 0.05 ml UK human anthrax vaccine together with 0.2 ml Ribi Trimix adjuvant, and two animals received physiological saline to act as controls.

Human anthrax vaccine is an alum-precipitated cell-free culture filtrate of *B. anthracis* strain 34F2, preserved with thiomersal. The culture supernatant from which the vaccine is made varies from lot to lot with respect to the concentration of PA, small contaminating quantities of LF and EF and other undefined bacterial and media constituents.

Ribi Trimix (M6661: Sigma Chemical Co, Poole, UK) is a synthetic adjuvant



formulation derived from bacterial components. It is a stable oil-in-water emulsion which can be used as an alternative to Freund's water-in-oil emulsion. Trimix is non-viscous (2% squalene oil-in-water emulsion) and easy to prepare. It is composed of 0.5 mg Monophosphoryl Lipid A (MPL<sup>®</sup>, highly-refined non-toxic Lipid A isolated from re-mutants of *Salmonella minnesota*), 0.5 mg synthetic Trehalose Dicorynomycolate (TDM, S-TDM, an analogue of trehalose dimycolate from the 'cord factor' of *Mycobacterium tuberculosis*) and cell wall skeleton (CWS, from deproteinised and delipidated cell walls of mycobacteria) in squalene and Tween 80 in water.

Prior to use, the adjuvant was warmed to 40 °C and reconstituted with warmed sterile saline. Control preparations were prepared using 2 ml sterile saline. Anthrax vaccine/Ribi preparations were prepared such that each dose (0.25 ml) consisted of 0.05 ml vaccine and 0.2 ml adjuvant reconstituted to manufacturers instruction.

Three weeks after the final vaccination, guinea pigs were carefully clipped and depilated on their backs on the preceding day. Prior to injection, each animal was marked with a spirit-based pen to show animal number and injection site. Injection sites were arranged so that each of the animals was injected intradermally with different levels of PA in 0.1 ml volumes of physiological saline. Saline solution was used as a negative control.

Native PA (nPA) was a gift from Dr P.C.B. Turnbull (CAMR, Porton Down, UK). It was produced from fermenter cultures of *B. anthracis* 34F2 strain. Fermenter culture was harvested via continuous flow centrifugation. The filtrate was concentrated using ultra-filtration. Proteins in the filtrate were precipitated using

ammonium sulphate. Following dialysis, crude protein was purified using fast protein liquid chromatography (FPLC).

Cutaneous reactions were measured by determining erythema and induration at 4, 24, 48 and 72 hours post intradermal injection. Erythema was measured as the diameter of the skin reaction. Induration was determined using a dial gauge to read the double skin thickness in millimetres. The results were averaged and the standard error of the mean calculated.

### **2.3.3 Lymphocyte proliferation assay**

During this investigation, two separate vaccination regimen were used. In the first, three groups of two guinea pigs were used. A group of guinea pigs was vaccinated at 0, 3 and 6 weeks with 0.05 ml UK human anthrax vaccine by intramuscular injection. Another group received UK human anthrax vaccine and Ribi Trimix adjuvant. A third control group received saline solution. In the second regimen, five groups of guinea pigs were vaccinated at 0, 2 and 4 weeks by intramuscular injection. Two animals received 0.05ml UK human anthrax vaccine. Two animals received UK human anthrax vaccine and Ribi Trimix adjuvant. Two animals received UK human anthrax vaccine, Ribi Trimix adjuvant and an extra 2.5µg nPA. Two animals received Ribi Trimix adjuvant and 2.5µg nPA. A control group of four animals received saline solution.

### *Blood and organ samples*

Guinea pigs, anaesthetised with diethyl ether, were bled by cardiac puncture and whole blood (20-25 ml) was placed into heparinised tubes. Once the animals had been killed by cervical dislocation, the spleens were removed and placed into 1-2 ml serum free RPMI 1640 tissue culture medium supplemented with 2 mM L-Glutamine solution (Gibco, Paisley, UK), 20 i.u/ml penicillin/streptomycin, and 25 mM HEPES buffer.

### *Lymphocyte separation from blood*

Heparinised blood was diluted with an equal volume of supplemented serum free RPMI 1640. Diluted blood (7 ml) was layered onto 3 ml Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) and centrifuged at 400g for 30 minutes.

The upper layer of medium and plasma was discarded. The monocytic cell cloud (buffy layer) was transferred to a clean tube. Supplemented serum free RPMI 1640 was added to fill the tube. Cells were centrifuged at 400g for 6 minutes. The supernatant was discarded and the pellet resuspended in supplemented serum free RPMI 1640.

### *Lymphocyte separation from whole spleen*

Spleens were rinsed in supplemented serum free RPMI 1640. The spleens were

teased apart using forceps, scalpel and hypodermic needles in 1 ml supplemented serum free medium. The tissue was gently forced through a tea strainer using a sterile syringe plunger to remove connective tissue and break up any large clumps of cells. The supplemented RPMI 1640 containing the spleen cells was placed in a clean tube and diluted 1:1 with like medium. The spleen cell mixture was layered onto 3 ml Ficoll-paque and lymphocytes separated as above.

Viable lymphocyte counts were determined as follows: Tubes were prepared containing 100  $\mu$ l Trypan blue solution and a 100  $\mu$ l aliquot of cell suspension was added to the tube. A 20  $\mu$ l aliquot of cell:trypan blue mixture was removed to a disposable haemocytometer for counting. Viable cells appeared as bright cells; non viable cells show blue staining. The cell concentration was adjusted to the required level (usually  $1 \times 10^6$  viable cells/ml).

### *Plate preparation*

Tissue culture (96-well) plates were prepared with 200  $\mu$ l RPMI 1640 plus L-glutamine in the outside (36) wells. Test agents, phytohaemagglutinin (PHA), Concanavalin A (Con A), Ribi adjuvant, nPA or alum (4% or 2%), were diluted in supplemented serum free RPMI 1640 and 4 replicates of 100  $\mu$ l of each dilution placed in the inner wells. An aliquot of cell suspension (100  $\mu$ l) was added to the inner wells. The plates were sealed and incubated under 5% CO<sub>2</sub> at 37°C for five days.

After five days, 1  $\mu$ Ci [<sup>3</sup>H]TdR (Specific Activity; 74 GBq mmol<sup>-1</sup>; Amersham) was

added to the inner 60 wells containing cells and the plates were returned to the incubator for a further 24 hours. Using a cell harvester (Skatron Inc, Virginia, USA) the plates were harvested onto glass fibre filters. The filters were dried at 37 °C for 2 hours, discs punched out that carried the dried cellular material, and placed in scintillation vials with 2ml Cytoscint ES (ICN Flow, Thane, UK). The samples were counted in a  $\beta$ -radiation counter for 1 minute. Results were corrected by subtracting the background count. The replicates of each treatment were averaged and the Stimulation Index calculated.

$$\text{Stimulation Index (SI)} = \frac{\text{Average count for test agent}}{\text{Average count for media control}}$$

A stimulation index of three or greater was considered a positive response (Surcel *et al.*, 1994).

#### 2.3.4 Aerosol challenge

All investigations involving *B. anthracis* spores and infected animals were carried out to the Advisory Committee on Dangerous Pathogens category III containment conditions (ACDP, 1995). The virulent Ames strain of *B. anthracis* was used during the aerosol challenge studies. The Ames strain was isolated from a bovine outbreak in Ames, Iowa, USA in 1980. The isolate is fully virulent (pXO1<sup>+</sup>; pXO2<sup>+</sup>) and lethal for guinea pigs (Little & Knudson, 1986). The isolate was cultured on blood agar with 5% horse blood for 24 hours. Colonies were transferred to sporulation agar slopes in 25 ml bottles and incubated for several days at 28 °C. The growth was washed off with 3-5 ml sterile distilled water (SDW) which was placed into a bottle and held at 62 °C for 15 minutes followed by subculture to check the purity of the

suspension. The 3-5 ml suspension of heat shocked spores were then used to inoculate new sporulation agar (Quinn *et al.*, 1990) in Roux bottles which were incubated for 2 weeks at 28 °C. The degree of sporulation was checked by phase contrast for the presence of >95% phase bright spores. The cells in each roux bottle were then washed off with 25-50 ml SDW. Spores were centrifuged and resuspended in 65% (v/v) isopropanol and held overnight at 4°C in the refrigerator. They were then centrifuged and resuspended in SDW three times, checked for purity and pure batches pooled, counted and stored at 4°C until required (Jones *et al.*, 1996b).

For aerosol challenge, spores were resuspended to a concentration of approximately  $10^9$  cfu/ml. An aerosol was generated by a collision nebuliser connected to a Henderson-Type apparatus and a nose-only exposure tube (Henderson, 1952; Druett, 1969). Guinea pigs were exposed to the aerosol for 7 minutes. All-glass impingers (AGI-30: May & Harper, 1957) were used to sample the aerosol and allow enumeration of the dose. Following challenge the animals were observed for 2 weeks (Jones *et al.*, 1996a).

## 2.4 Results

### 2.4.1 Delayed-type hypersensitivity skin test

Table 2.1 presents the results of the cutaneous response 4 hours after intradermal challenge. Erythema was noted in the groups vaccinated with human vaccine or human vaccine and Ribi adjuvant at all challenge levels of nPA. As the challenge increased, so did the degree of erythema noted. Erythema in animals vaccinated with human anthrax vaccine (V) showed a significant difference to control animals (C) after intradermal injection of 10 µg nPA ( $P \leq 1\%$ ). Animals vaccinated with human anthrax vaccine and Ribi adjuvant (VR) showed significant differences to control animals after injection of 1 and 10 µg nPA ( $P \leq 2.5$  and 5 %, respectively). However, statistical analyses of VR and V groups showed no significant difference between the groups.

Changes in induration after vaccination were less pronounced. Significant differences occurred between both test groups when compared to the control at challenge doses of 0 and 10 µg nPA ( $P \leq 2.5$  and 5 %). Analyses of VR and V groups again showed no significant difference between them.

After 24 hours, the levels of erythema increased in the VR test group, but not in the V test group (Table 2.2). Analysis of the results comparing test groups with control and test groups against each other showed no significant differences between any of the groups.

In terms of induration, the V test group showed a significantly different response to

Vaccine Group	Animal No.	Erythema (mm <sup>2</sup> )				Induration (mm)			
		Protective Antigen (µg)				Protective Antigen (µg)			
		0	0.1	1	10	0	0.1	1	10
Vaccine	1	0	12.5	75.0	137.5	4.00	4.18	4.98	5.58
	2	0	25.0	12.5	52.0	4.25	4.40	4.80	5.75
	3	0	0	0	222	3.73	3.15	3.95	5.30
	4	0	0	25.0	274.5	4.10	4.00	4.30	4.85
	Mean ± S.E.M	0 ± 0	9.4 ± 5.2	28.1 ± 14.2	171.5 ± 42.2	4.02 ± 0.10	4.08 ± 0.12	4.51 ± 0.20	5.37 ± 0.17
Vaccine + Ribi	5	0	7.5	50.0	187.5	3.60	3.65	3.95	5.20
	6	0	12.5	25.0	50.0	3.60	4.35	3.90	4.75
	7	0	0	25.0	450	4.60	4.60	5.25	6.25
	8	0	0	0	125	3.50	3.60	4.00	5.50
	Mean ± S.E.M	0 ± 0	5 ± 2.7	25 ± 8.8	203.1 ± 75.3	3.83 ± 0.22	4.05 ± 0.22	4.28 ± 0.28	5.43 ± 0.27
Control	9	0	0	0	0	4.75	4.25	4.60	4.50
	10	0	0	0	0	4.55	4.35	4.00	4.65
	Mean ± S.E.M	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4.65 ± 0.07	4.30 ± 0.04	4.30 ± 0.21	4.58 ± 0.05
Statistical Analysis *									
V - C (DF 4)	NS	NS	NS	NS	1 %	2.5 %	NS	NS	2.5 %
VR - C (DF 4)	NS	NS	NS	2.5 %	5 %	2.5 %	NS	NS	5 %
VR - V (DF 6)	NS	NS	NS	NS	NS	NS	NS	NS	NS

N.B. Mean ± Standard Error of the Mean (S.E.M.); \* P values for differences between groups in terms of erythema and induration were determined by Student's t test.  
DF, Degrees of Freedom; n<sub>1</sub>+n<sub>2</sub>-2; NS, not significant.

**Table 2.1** Cutaneous response 4 Hours after intradermal injection of different amounts of Protective Antigen.



Vaccine Group	Animal No.	Erythema (mm <sup>2</sup> )				Induration (mm)			
		Protective Antigen (µg)				Protective Antigen (µg)			
		0	0.1	1	10	0	0.1	1	10
Vaccine	1	0	7.5	78.0	115	3.95	3.90	4.45	5.00
	2	0	25.0	0	75.0	4.00	4.45	4.55	4.90
	3	0	0	10.0	110	3.60	3.90	3.80	4.25
	4	0	0	27.5	239	3.80	3.850	3.80	5.10
	Mean ± S.E.M	0 ± 0	8.10 ± 5.1	28.9 ± 15.0	134.8 ± 31.1	3.84 ± 0.08	4.03 ± 0.12	4.18 ± 0.16	5.37 ± 0.16
Vaccine + Ribi	5	0	53.0	52.0	187.5	3.35	3.50	3.50	4.60
	6	0	27.0	15.0	474.5	4.25	4.55	4.80	5.80
	7	0	5.0	59.5	450	4.60	4.60	5.25	6.25
	8	0	0	0	125	3.40	3.45	3.70	4.95
	Mean ± S.E.M	0 ± 0	21.3 ± 10.5	31.6 ± 12.4	253 ± 66.4	3.76 ± 0.20	4.00 ± 0.26	4.15 ± 0.28	5.15 ± 0.22
Control	9	0	0	0	54.0	4.40	4.50	4.85	4.40
	10	0	0	0	0	4.20	4.25	4.10	4.70
	Mean ± S.E.M	0 ± 0	0 ± 0	0 ± 0	27 ± 19.1	4.30 ± 0.07	4.38 ± 0.09	4.48 ± 0.27	4.55 ± 0.11
Statistical Analysis *									
V - C (DF 4)	NS	NS	NS	NS	NS	2.5 %	NS	NS	2.5 %
VR - C (DF 4)	NS	NS	NS	NS	NS	NS	NS	NS	NS
VR - V (DF 6)	NS	NS	NS	NS	NS	NS	NS	NS	NS

N.B. Mean ± Standard Error of the Mean (S.E.M.); \* P values for differences between groups in terms of erythema and induration were determined by Student's t test.  
DF, Degrees of Freedom; n<sub>1</sub>+n<sub>2</sub>-2; NS, not significant.

**Table 2.2** Cutaneous response 24 Hours after intradermal injection of different amounts of Protective Antigen.

the control animals at 0 and 10 µg nPA challenge doses ( $P \leq 2.5\%$ ). Comparison of VR to C, and VR to V animals gave no significant differences between different vaccination regimen.

The different vaccination groups showed a reduced level of erythema at 48 hours post-challenge compared to 24 hours. The results for 48 hours after intradermal challenge are presented in Table 2.3. The only significant difference between the groups was between VR and C animals after 10 µg nPA ( $P \leq 1\%$ ).

Measurement of induration showed significant differences between V and C groups of animals at doses of 0 and 0.1 µg nPA ( $P \leq 2.5\%$  and  $1\%$ , respectively). At this point, the levels of induration noted for the test animals had actually fallen below that of the control group animals. All other groups showed no significant differences.

After 72 hours all incidences of erythema had resolved (Table 2.4), thus there were no significant differences between different vaccination regimen.

Results for induration measurement showed that there were significant differences between V and C animals at 0 and 0.1 µg nPA ( $P \leq 2.5\%$ ). All other differences between groups were not significant.

Vaccine Group	Animal No.	Erythema (mm <sup>2</sup> )				Induration (mm)			
		Protective Antigen (µg)				Protective Antigen (µg)			
		0	0.1	1	10	0	0.1	1	10
Vaccine	1	0	0	0	75.0	4.00	3.80	4.20	4.55
	2	0	0	0	0	4.05	4.20	4.20	4.15
	3	0	0	0	0	3.70	3.65	3.65	3.75
	4	0	0	0	0	3.65	3.55	3.60	4.10
	Mean ± S.E.M	0 ± 0	0 ± 0	0 ± 0	18.8 ± 16.2	3.85 ± 0.09	3.80 ± 0.12	3.93 ± 0.15	4.14 ± 0.14
Vaccine + Ribi	5	0	0	0	37.5	3.30	3.15	3.10	3.90
	6	0	8.0	36.0	48.0	3.90	4.15	4.30	4.55
	7	0	0	37.5	147.5	4.20	4.30	4.25	4.65
	8	0	0	0	77.0	3.40	3.25	3.55	4.15
	Mean ± S.E.M	0 ± 0	2 ± 1.7	18.4 ± 9.2	77.5 ± 21.5	3.70 ± 0.18	3.71 ± 0.26	3.80 ± 0.25	4.31 ± 0.15
Control	9	0	0	0	0	4.25	4.3	4.95	4.1
	10	0	0	0	0	4.35	4.3	4.1	4.6
	Mean ± S.E.M	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4.30 ± 0.04	4.30 ± 0	4.53 ± 0.30	4.35 ± 0.18
Statistical Analysis *									
V - C (DF 4)	NS	NS	NS	NS	NS	2.5 %	1 %	NS	NS
VR - C (DF 4)	NS	NS	NS	NS	1 %	NS	NS	NS	NS
VR - V (DF 6)	NS	NS	NS	NS	NS	NS	NS	NS	NS

N.B. Mean ± Standard Error of the Mean (S.E.M.); \* P values for differences between groups in terms of erythema and induration were determined by Student's t test.  
DF, Degrees of Freedom; n<sub>1</sub>+n<sub>2</sub>-2; NS, not significant.

**Table 2.3** Cutaneous response 48 Hours after intradermal injection of different amounts of Protective Antigen.

Vaccine Group	Animal No.	Erythema (mm <sup>2</sup> )					Induration (mm)				
		Protective Antigen (µg)					Protective Antigen (µg)				
		0	0.1	1	10	0	0.1	1	10	0	10
Vaccine	1	0	0	0	0	3.95	3.75	4.20	4.30	3.95	4.30
	2	0	0	0	0	3.85	4.15	4.20	4.00	3.85	4.00
	3	0	0	0	0	3.55	3.50	3.60	3.75	3.55	3.75
	4	0	0	0	0	3.35	3.65	3.75	3.95	3.35	3.95
	Mean ± S.E.M	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3.68 ± 0.12	3.76 ± 0.12	3.94 ± 0.13	4.0 ± 0.10	3.68 ± 0.12	4.0 ± 0.10
Vaccine + Ribi	5	0	0	0	0	3.30	3.15	3.20	3.75	3.30	3.75
	6	0	0	0	0	4.00	4.10	4.20	3.85	4.00	3.85
	7	0	0	0	0	4.45	4.55	4.85	4.90	4.45	4.90
	8	0	0	0	0	3.55	3.55	3.7	4.05	3.55	4.05
	Mean ± S.E.M	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3.83 ± 0.22	3.84 ± 0.27	4 ± 0.31	4.15 ± 0.23	3.83 ± 0.22	4.15 ± 0.23
Control	9	0	0	0	0	4.25	4.35	4.45	4.05	4.25	4.05
	10	0	0	0	0	4.15	4.20	4.15	4.65	4.15	4.65
	Mean ± S.E.M	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4.2 ± 0.04	4.28 ± 0.05	4.30 ± 0.11	4.35 ± 0.21	4.2 ± 0.04	4.35 ± 0.21
Statistical Analysis *											
V - C (DF 4)	NS	NS	NS	NS	NS	2.5 %	2.5 %	NS	NS	NS	NS
VR - C (DF 4)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
VR - V (DF 6)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

N.B. Mean ± Standard Error of the Mean (S.E.M.); \* P values for differences between groups in terms of erythema and induration were determined by the Student's t test. DF, Degrees of Freedom; n<sub>1</sub>+n<sub>2</sub>-2; NS, not significant.

**Table 2.4** Cutaneous response 72 Hours after intradermal injection of different amounts of Protective Antigen

#### 2.4.2 Lymphocyte proliferation assay

Initially, a comparison was to be made between the response of guinea pigs immunised with vaccine plus Ribi adjuvant (VR), and those vaccinated with vaccine alone (V). The viable cell counts for blood lymphocytes from the VR and V animals were  $7.2 \times 10^6$  and  $2 \times 10^7$  cells per ml respectively. After separation of spleen cells, it was found that the yield was not sufficient to allow cells to be used for the assay, thus two plates were used for the blood separated lymphocytes. The plates were seeded at  $1 \times 10^5$  viable cells/well. The alum being tested as one of the reagents blocked the harvester jets leading to cross-contamination between wells on the plates. The cross-contamination led to the results being disregarded due to questionable accuracy. The decision was made to stop using alum and use the extra wells to test different concentrations of Ribi adjuvant and ConA.

In the second part of the investigation, again using animals from the first regimen, a comparison was made of the response of VR and saline control (C) animals. Cell counts for blood and spleen derived lymphocytes from the C animals were  $5.1 \times 10^7$  and  $1.9 \times 10^7$  cells per ml respectively. VR animals showed cell counts of  $5.5 \times 10^7$  and  $8.2 \times 10^6$  cells per ml respectively. The wells were seeded at  $2 \times 10^5$  viable cells/well. The low yield of spleen cells from the VR animal meant that stimulation with protective antigen at 25 and 12.5  $\mu\text{g/ml}$  were not tested and all other treatments were done as triplicates. The stimulation index results are shown in Table 2.5.

Stimulant	Concentration	Vaccine Preparation			
		Vaccine + Ribl		Saline Control	
		Blood Lymphocyte	Spleen Lymphocyte	Blood Lymphocyte	Spleen Lymphocyte
Con A (µg/ml)	10	*	9.6	128	144.2
	5	1.70	8.3	24.9	62.4
	2.5	0.40	1.8	0.44	1.86
PHA (µg/ml)	2	0.29	5.9	23.3	40.6
	1	0.07	14	-0.52	81
Ribi (µg/ml)	50	0.17	0.08	0.27	0.20
	10	0.14	0.3	0.77	2.6
	2.5	0.34	0.7	-1.18	1.43
Protective	50	0.68	0.4	104.4	0.07
Antigen (µg/ml)	25	0.64	*	0.38	0.14
	12.5	0.34	*	417.7	1.31

Note: \* No result; Results shown as means of three or four readings. Concentration shown as final concentration in test well. The results are given as the calculated stimulation index. Stimulation index greater than three shows proliferation.

**Table 2.5** *In vitro* Lymphocyte proliferation response of guinea pig blood and spleen-derived lymphocytes exposed to native PA, mitogens and Ribi adjuvant

Spleen derived lymphocytes (SDL) showed greater proliferation to the control mitogens, Con A and PHA, than blood derived lymphocytes (BDL). BDL from the VR animal showed no proliferation (Stimulation index  $\leq 3$ ). This suggests the BDL did not survive the isolation and separation procedures, unlike the SDL. The SDL did not proliferate in response to the test antigens, Ribi adjuvant and nPA.

Both BDL and SDL from the C animal showed proliferation to the control mitogens. BDL did not proliferate due to Ribi adjuvant. However, BDL gave an SI of 104.4 and 417.7 for 50 and 12.5  $\mu\text{g/ml}$  of nPA, respectively. This is an unexpected response from an animal that was not vaccinated with nPA. It was assumed that this was a non-specific response and may be due to trace amounts of mitogenic contaminants in the nPA used for the proliferation assay. However, lethal toxin has been shown to be mitogenic for human T-cells (Guidi-Rontani *et al.*, 1997). SDL did not show proliferation to Ribi or nPA.

The lymphocyte proliferation response of vaccine alone was compared to the response of a saline control animal. Again these animals were vaccinated under the first regimen. In this experiment for preparation of the lymphocyte proliferation assay plates, the cell counts were  $2.3 \times 10^7$  and  $2.1 \times 10^7$  for BDL from the V and C animals. SDL were  $2.7 \times 10^6$  and  $9.3 \times 10^5$ . Lymphocytes derived from peripheral blood were seeded at  $2 \times 10^5$  viable cells/well. However, due to insufficient cells from the spleen separations, SDL were seeded at  $1 \times 10^5$ . The only reagents that could be tested for the C immunised animal were media and nPA (50  $\mu\text{g/ml}$ ). Media, Con A (10  $\mu\text{g/ml}$ ), Ribi (50  $\mu\text{g/ml}$ ) and nPA (50  $\mu\text{g/ml}$ ) were the only reagents tested against SDL from the V animal. All tests were done as replicates of four. The

stimulation index results are shown in Table 2.6.



Stimulant	Concentration	Vaccine Preparation			
		Vaccine		Saline	
		Blood Lymphocyte	Spleen Lymphocyte	Blood Lymphocyte	Spleen Lymphocyte
Con A ( $\mu\text{g/ml}$ )	10	2.1	25.1	2.15	*
	5	*	*	1.21	*
	2.5	0.25	*	0.59	*
PHA ( $\mu\text{g/ml}$ )	2	2.1	*	9.21	*
	1	2.4	*	11.39	*
Ribi ( $\mu\text{g/ml}$ )	50	0.14	0.06	1.03	*
	25	1.11	*	5.77	*
	12.5	0.39	*	0.69	*
Protective Antigen ( $\mu\text{g/ml}$ )	50	0.06	0.03	0.51	0.03
	25	0.04	*	0.41	*
	12.5	0.12	*	0.51	*

N.B.\* No result; Results shown as means of three or four readings. Concentration shown as final concentration in test well. Stimulation index greater than three shows proliferation.

**Table 2.6** Stimulation indices of guinea pig blood and spleen-derived lymphocytes exposed to native PA, mitogens and Ribi adjuvant

BDL from the V animal showed no proliferation to any of the test antigens or control mitogens. SDL showed proliferation to Con A indicating survival after isolation and separation procedures. No proliferation was seen at the levels of Ribi and nPA tested.

The SDL from the C animal showed no proliferative response against nPA, the only antigen tested. BDL showed a proliferative response to the PHA (control mitogen) and 25 µg/ml Ribi adjuvant. The response to Ribi was not expected because Ribi was not included in the vaccination regimen of this animal. Ribi adjuvant is composed of a number of salmonella and mycobacterial-derived antigens. The control animals may have been showing a recall response to epitopes present on strains of salmonella and mycobacteria in the environment and Ribi adjuvant.

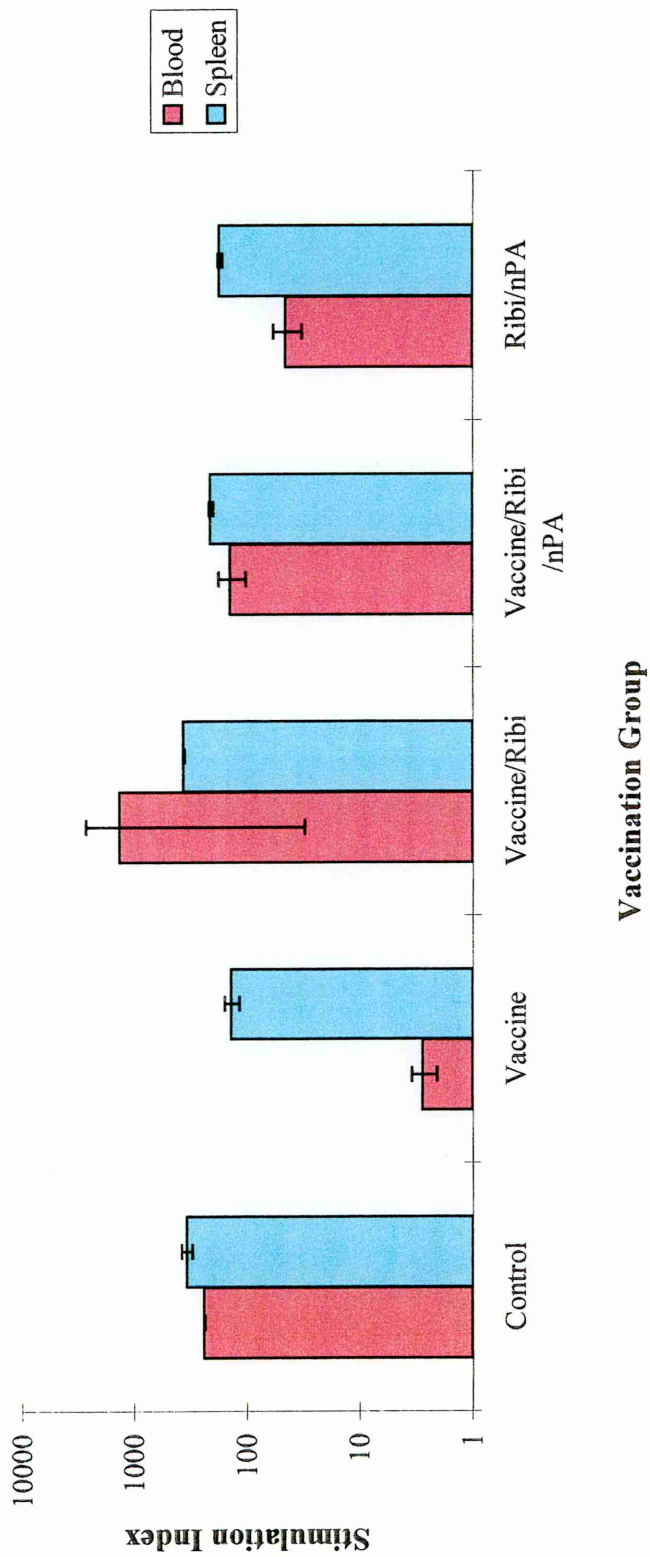
The lymphocyte proliferative response of cells taken from animals vaccinated under the second regimen were compared (Table 2.7). Cell counts were completed for BDL (average  $2.1 \times 10^7 \pm 2.1 \times 10^6$ ; range  $1 \times 10^7$  to  $3.1 \times 10^7$ ) and SDL (average  $3.3 \times 10^7 \pm 2.7 \times 10^6$ ; range  $1.5 \times 10^7$  to  $5 \times 10^7$ ). The wells were seeded at  $1 \times 10^5$  cells/well.

Con A caused proliferation in BDL from all the vaccine groups, except animals receiving vaccine alone (Figure 2.1). PHA caused proliferation in BDL from all

Stimulant	Concentration	Vaccination Regimen									
		Control		vaccine		Vaccine + Ribi		Vaccine + Ribi + nPA		Ribi + nPA	
		Blood	Spleen	Blood	Spleen	Blood	Spleen	Blood	Spleen	Blood	Spleen
ConA ( $\mu\text{g/ml}$ )	10	242.2 $\pm$ 2.1	345.6 $\pm$ 36.1	2.8 $\pm$ 0.7	138 $\pm$ 19.4	1352.9 $\pm$ 1321.8	367.5 $\pm$ 3.7	140 $\pm$ 37.5	208.7 $\pm$ 8.4	45.5 $\pm$ 12.7	174.5 $\pm$ 7.5
PHA ( $\mu\text{g/ml}$ )	1	242.2 $\pm$ 378.3	137.4 $\pm$ 34.5	150.6 $\pm$ 140	68.7 $\pm$ 51.5	1026.9 $\pm$ 803.3	116.8 $\pm$ 13.3	14.1 $\pm$ 2.3	108.5 $\pm$ 41.3	44.1 $\pm$ 7.5	47 $\pm$ 27.6
Ribi ( $\mu\text{g/ml}$ )	50	0.4 $\pm$ 0.1	0.06 $\pm$ 0.02	0.2 $\pm$ 0.1	0.05 $\pm$ 0.02	0.4 $\pm$ 0.3	0.02 $\pm$ 0	0.4 $\pm$ 0.2	0.02 $\pm$ 0.01	0.3 $\pm$ 0.3	0.03 $\pm$ 0.02
	5	5.7 $\pm$ 2.9	3.2 $\pm$ 0.9	1.7 $\pm$ 0.9	0.9 $\pm$ 0.2	175.7 $\pm$ 147.1	3.1 $\pm$ 0.8	19.6 $\pm$ 15.8	3.4 $\pm$ 1.8	3.9 $\pm$ 0.3	1.2 $\pm$ 0.5
	0.5	5.1 $\pm$ 1.9	1.6 $\pm$ 0.3	2.2 $\pm$ 0.9	0.7 $\pm$ 0.05	203.6 $\pm$ 177.4	2.8 $\pm$ 1.2	24.0 $\pm$ 20.5	3.5 $\pm$ 1.9	6.5 $\pm$ 2.1	0.6 $\pm$ 0.05
nPA ( $\mu\text{g/ml}$ )	50	0.6 $\pm$ 0.2	0.3 $\pm$ 0.06	2.1 $\pm$ 2.0	0.4 $\pm$ 0.4	37.8 $\pm$ 33.8	1.0 $\pm$ 0.3	5.2 $\pm$ 4.8	2.0 $\pm$ 0.6	3.7 $\pm$ 3.6	1.0 $\pm$ 1.0
	25	2.1 $\pm$ 1.0	0.4 $\pm$ 0.1	1.1 $\pm$ 1.1	0.4 $\pm$ 0.2	87.4 $\pm$ 75.9	1.9 $\pm$ 1.1	20.1 $\pm$ 19.0	2.7 $\pm$ 0.4	9.7 $\pm$ 6.3	1.6 $\pm$ 1.1
	12.5	6.0 $\pm$ 5.4	0.3 $\pm$ 0.06	1.1 $\pm$ 1.0	0.2 $\pm$ 0.1	100.4 $\pm$ 4.1	1.1 $\pm$ 0.7	27.0 $\pm$ 25.0	2.7 $\pm$ 0.4	17.7 $\pm$ 5.4	1.8 $\pm$ 1.4

Note: Concentration shown as final concentration in test well. Stimulation Index stated as mean  $\pm$  Standard error of mean; Stimulation index greater than three shows proliferation. However, SEM in some cases exceeds the mean indicating variability within the group.

**TABLE 2.7** Summary of results for stimulation indices of blood and spleen-derived guinea pig lymphocytes exposed to native PA, mitogens and Ribi adjuvant

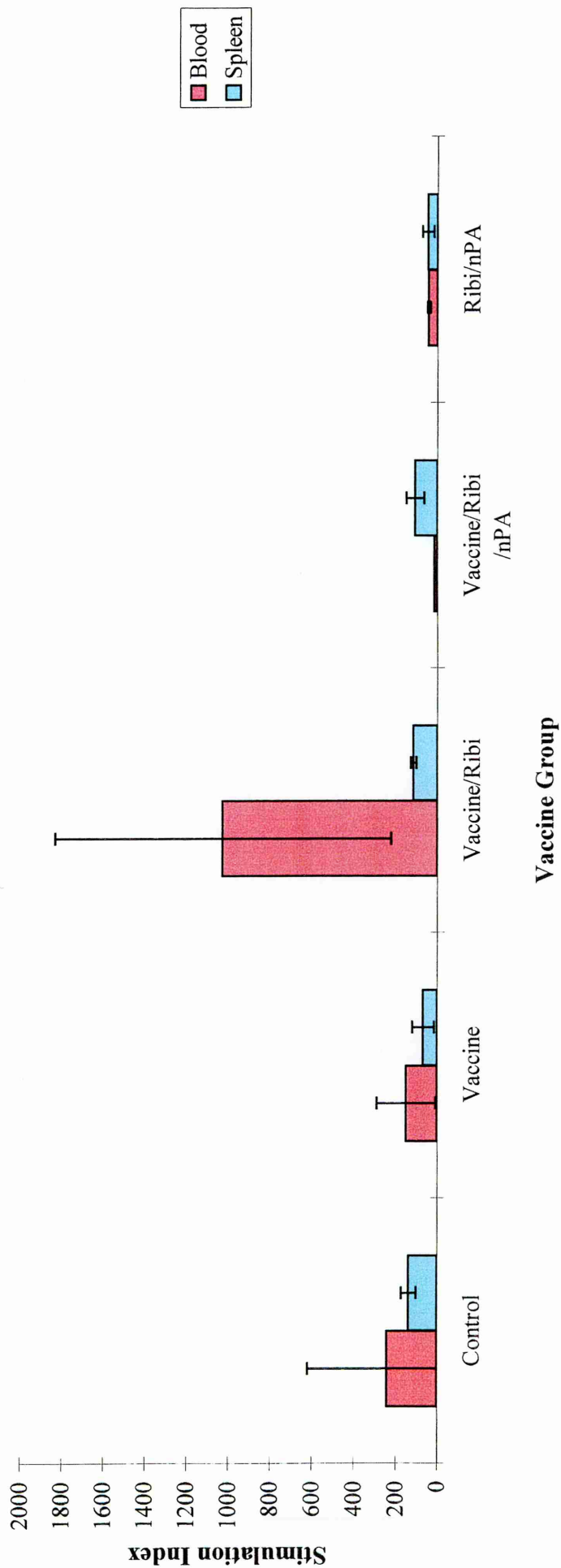


**Figure 2.1** Concanavalin A induced proliferation by guinea pig lymphocytes

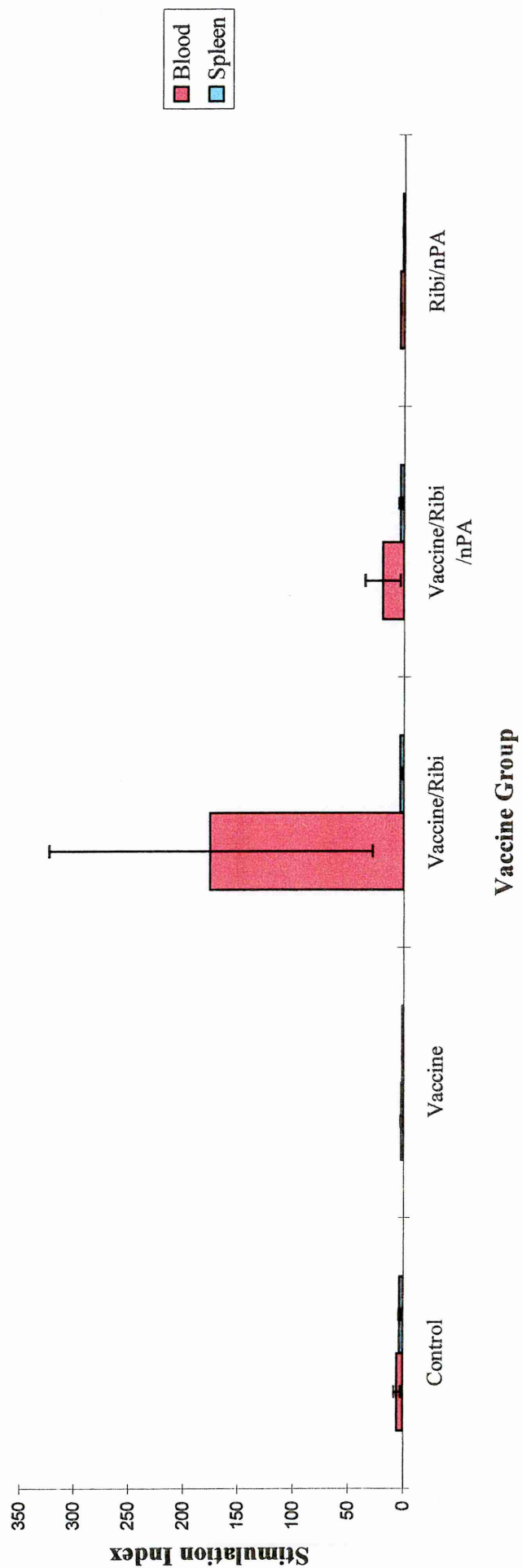
vaccine groups (Figure 2.2). A proliferation response to Ribi adjuvant was seen for BDL from the C, VR, vaccine Ribi nPA (VRP), and Ribi nPA (RP) groups (Figure 2.3). Again, the control animals may have developed a recall response to environmental strains of salmonella and mycobacteria that possess epitopes present in Ribi adjuvant. nPA caused proliferation in BDL from VR, VRP and RP animals. The control animals showed some proliferation at 12.5 µg/ml (Figure 2.4). This is presumed to be due to trace amounts of mitogenic contamination such as the mitogenic effect of contaminating lethal factor. Animals immunised with V showed no proliferation in response to nPA.

The responses for BDL, in terms of proliferation, to Ribi and nPA by the different groups was compared using a Student's t-test (Table 2.8). Significant differences ( $P \leq 0.5\%$ ) were seen for BDL exposed to 12.5 µg/ml in the following comparisons; VR to C, VR to V and VR to RP.

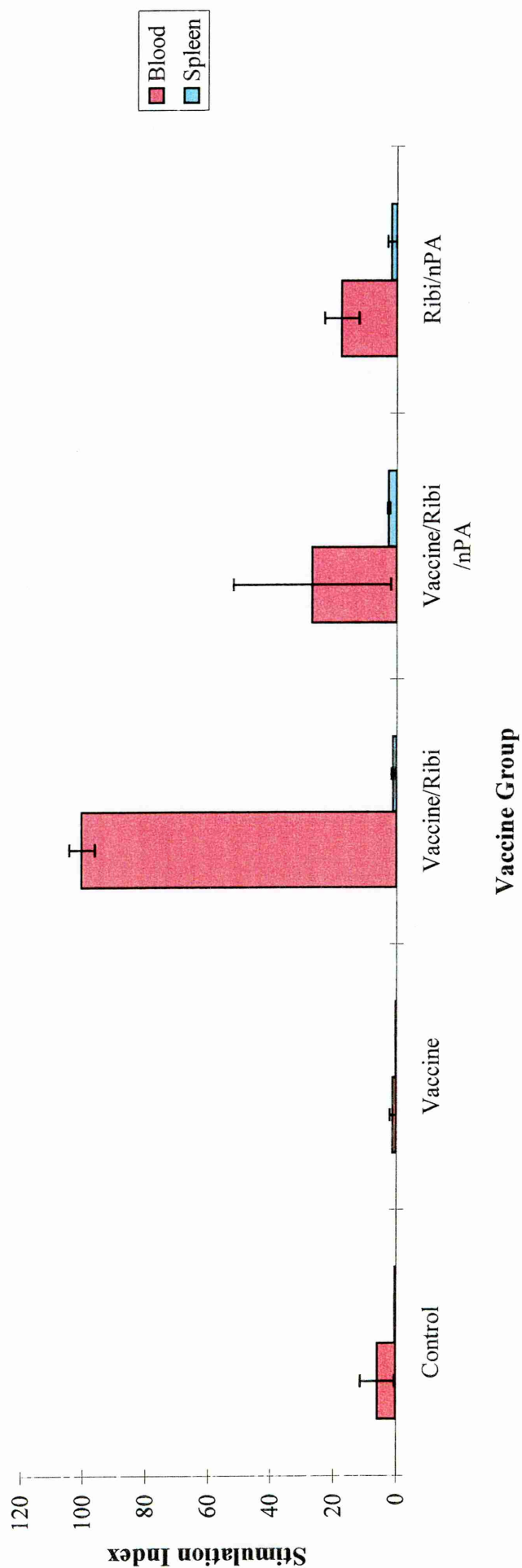
The control mitogens, Con A and PHA stimulated proliferation in SDL from all groups of animals (Table 2.7), thus indicating that the cells had survived isolation and separation. Ribi adjuvant caused proliferation in SDL from VRP animals at 5 and 0.5 µg/ml. Ribi adjuvant at 5 µg/ml caused proliferation in SDL from C, VR and VRP groups. Again, presence of a recall response was thought to be due to epitopes present in Ribi adjuvant and on strains of salmonella and mycobacteria in the environment.



**Figure 2.2** Phytohemagglutinin induced proliferation by guinea pig lymphocytes



**Figure 2.3** Lymphocyte proliferation after stimulation with 5 µg/ml Ribi adjuvant



**Figure 2.4** Lymphocyte proliferation after stimulation with 12.5  $\mu\text{g/ml}$  native PA



Comparison (DF)	Test Antigen					
	Ribi Trimix (µg/ml)			*Protective Antigen (µg/ml)		
	50	5	0.5	50	25	12.5
V-C (4)	NS	NS	NS	NS	NS	NS
VR-C (4)	NS	NS	NS	NS	NS	P≤0.5%
VRP-C (4)	NS	NS	NS	NS	NS	NS
RP-C (4)	NS	NS	NS	NS	NS	NS
VR-V (2)	NS	NS	NS	NS	NS	P≤0.5%
VRP-V (2)	NS	NS	NS	NS	NS	NS
RP-V (2)	NS	NS	NS	NS	NS	NS
VRP-VR (2)	NS	NS	NS	NS	NS	NS
RP-VR (2)	NS	NS	NS	NS	NS	P≤0.5%
RP-VRP (2)	NS	NS	NS	NS	NS	NS

N.B. \* P values for differences between groups were determined by the Student's t test.

DF, Degrees of Freedom;  $n_1+n_2-2$ ; NS, not significant.

**Table 2.8** Statistical comparison of blood-derived lymphocytes from the different experimental groups

Comparison (DF)	Test Antigen					
	Ribi Trimix (µg/ml)			*Protective Antigen (µg/ml)		
	50	5	0.5	50	25	12.5
V-C (4)	NS	NS	NS	NS	NS	NS
VR-C (4)	NS	NS	NS	NS	NS	NS
VRP-C (4)	NS	NS	NS	NS	P≤1%	P≤0.5%
RP-C (4)	NS	NS	NS	NS	NS	NS
VR-V (2)	NS	NS	NS	NS	NS	NS
VRP-V (2)	NS	NS	NS	NS	NS	P≤2.5%
RP-V (2)	NS	NS	NS	NS	NS	NS
VR-VRP (2)	NS	NS	NS	NS	NS	NS
RP-VR (2)	NS	NS	NS	NS	NS	NS
RP-VRP (2)	NS	NS	NS	NS	NS	NS

N.B. \* P values for differences between groups were determined by the Student's t test.

DF, Degrees of Freedom;  $n_1+n_2-2$ ; NS, not significant.

**Table 2.9** Statistical comparison of spleen-derived lymphocytes from the different experimental groups

Animals from the V and RP groups showed no proliferation at any of the levels of Ribi adjuvant tested. nPA did not stimulate proliferation in SDL from any of the groups. However, comparison of the SDL responses from the different groups using a Student's t-test (Table 2.9) showed significant differences ( $P \leq 1\%$  and  $0.5\%$ ) for VRP compared to C at 25 and 12.5  $\mu\text{g/ml}$  nPA. A significant difference was found when VRP was compared to V at 25  $\mu\text{g/ml}$  nPA ( $P \leq 2.5\%$ ).

#### **2.4.3 Aerosol challenge of vaccinated animals and comparison to lymphocyte proliferation results.**

The animals from different vaccination groups were challenged with a retained lung dose of  $10^6$  spores (Jones *et al.*, 1996a). Table 2.10 shows the survivors after 14 days from the different vaccination groups.

The results from the aerosol challenge show that vaccine alone does not fully protect against inhalational anthrax in a guinea pig model. Vaccine supplemented by Ribi adjuvant alone, and in combination with increased nPA raises the protection. The best vaccination regimen was shown to be a formulation of Ribi adjuvant and purified nPA.

If the results for the lymphocyte proliferation assay (Table 2.8 and 2.9) are compared to the aerosol challenge data (Table 2.10), the lymphocyte assay would suggest that the VR or VRP animals would survive best (Table 2.8 and 2.9). Challenge data shows that they are from some of the better groups, but animals vaccinated with Ribi and nPA survive best. RP animals did not show any significant differences in terms of

proliferation compared to the other vaccine groups.

Vaccine Group	% Survivors after 14 days (No. Survivors/Total No. challenged)
Saline	0% (0/9)
Vaccine only	11% (1/9)
Vaccine + Ribi adjuvant	90% (9/10)
Vaccine + Ribi adjuvant + 2.5 µg nPA	87.5% (7/8)
Ribi adjuvant + 2.5 µg nPA	100% (8/8)

**Table 2.10** Survivors 14 days after challenge with an aerosol of *B. anthracis*  
Ames spores

## 2.5 Discussion

Differences in terms of erythema and induration occurred in guinea pigs vaccinated with two different vaccination regimen. However, the majority of differences are not statistically significant at the level of  $P \leq 5\%$ . Erythema was seen in groups, vaccinated with VR, and V alone, after exposure to different levels of nPA at 4 hours post injection (Table 2.1). The erythema increased with increasing nPA. Induration was less pronounced because cellular recruitment would not have taken place in such a short time period.

After 24 hours, the levels of erythema decrease in the V group, but continue to increase in the VR group (Table 2.2). Although, measurement of induration showed no great change, animals from the V group showed a significant difference compared to the C group. A reduction of erythema had occurred by 48 hours. Significant differences were evident between VR and C (Table 2.3). However, induration measurements show a reduction compared to the C group.

Resolution of the erythema happened by 72 hours post-injection. The results for induration show that there was no widespread increase in skin thickness. Induration in the vaccinated groups was actually lower than that of the control animals.

The early changes noted after the DTH skin test of vaccinated animals may be due to the Arthus reaction. Circulating antibody binds to injected antigen leading to formation of immune complexes (antibody/antigen complex). Immune complexes can lead to an inflammatory response at the site of deposition. The response arises because of a number of factors, including complement and macrophage activation,

and platelet aggregation.

The continued increase in erythema at 24 hours for the VR group could be due to recruitment of immune effector cells, such as activated macrophages, to the injection site. The significant difference between V and C groups in terms of induration could be explained by greater inflammatory responses at the site due to presence of antibody.

At 48 hours, the reduction in erythema could be due to a reduction in the local concentration of available antibody and removal of deposited immune complexes. Significant differences between VR and C animals may be due to recruitment of effector cells and/or presence of antibody/antigen complexes. The reduction of induration in vaccinated animals compared to control animals suggests that cellular recruitment may not be involved.

Reduction of erythema by 72 hours could be due to removal of antigen by immune complex formation with antibody, or by degradation of antigen by antigen-presenting or immune effector cells. The immune response would reduce in light of a reduction in antigen levels. Induration measurements show no increase suggesting that immune effector cells were not recruited to the site of injection in any great numbers.

The results for the assay suggest that DTH does not occur in guinea pigs following the vaccination regimen used in this study. Histological examination would have been a useful tool to provide evidence for DTH. Accumulation of mononuclear cells around small veins followed by infiltration of the dermis can be seen by histology. In reflection, the group sizes were small for an outbred population of animals. The sizes

were a function of availability and logistics within the guinea pig facility. Presence of genetic variation may be at a sufficient level to obscure the results of the investigation. In addition, DTH is difficult to measure and ideally should be carried out objectively and 'blinded'.

Initial investigations using a lymphocyte proliferation assay showed some success in obtaining cells that could proliferate after stimulation with the control mitogens. There were some problems with isolation and separation that led to insufficient cell numbers for a full assay to be completed. This problem was overcome in later investigations by further use of isolation techniques.

Investigation of lymphocytes derived from the blood of animals vaccinated by different regimen showed some significant differences (Table 2.8). Lower variation between the animals would have increased the number of significant differences. Proliferation responses were extremely variable between animals and may reflect the outbred nature of Dunkin-Hartley guinea pigs. It may be that outbred animals show variation in the time to maximum response and concentration of nPA used to stimulate cultures. The short period from final boost to assay may also influence the results. Presence of other factors, such as LF and EF in the nPA solution used in the assay may be toxic to cells. This could mean that antigen-presentation may be reduced leading to a lower proliferative response. In a study by Guidi-Rontani and others (1997), presence of lethal toxin has been reported to be mitogenic to human T-cells. This may explain the occurrence of some level of proliferation in unimmunised animals. Results from the aerosol challenge experiment could not be correlated with results from the proliferation assay. If the genetic variability within the guinea pigs and inherent variability of the proliferation assay could be reduced



correlation may be seen. The investigation has shown that a memory response can be induced by vaccination of guinea pigs.

The hypothesis that CMI is responsible for protection against anthrax infection has not been confirmed. Investigations of the role cell-mediated and humoral immunity play in protection against infection will continue using a murine model. The use of a murine model will allow elucidation of the role of different immunoglobulin subclasses and cellular proliferation responses in protection against *B. anthracis* infection.

## CHAPTER 3.0

Investigation of the immune response  
to anthrax vaccination using a mouse model

### **3.0 Investigation of the immune response to anthrax vaccination using a mouse model**

#### **3.1 Introduction**

##### **3.1.1 Murine models and anthrax investigations**

Mice were originally used to detect *B. anthracis* and diagnose livestock infections (Topley & Wilson, 1931; Sterne, 1959). Inbred mice were used to differentiate between *B. anthracis* strains in the late 1970's (Abalakin & Cherkasski, 1978). Mice have also been utilised to investigate the pathogenesis of *B. anthracis*. Early studies of inhalational anthrax (Buchner, 1888) were extended by workers in the USA, where the development of an early whole body aerosol exposure apparatus allowed the investigation of the relative resistance of different animal species to *B. anthracis* infection (Young *et al.*, 1946). Barnes (1947b) studied inhalational anthrax in a mouse model using a head-only exposure apparatus. It was found that higher numbers of *B. anthracis* spores were required to initiate an inhalational infection compared to subcutaneous infection.

The action of toxin *in vivo* was investigated using a murine model (Lincoln *et al.*, 1967). The toxicity of purified PA and LF and the contribution of the individual toxin components to virulence has been assessed using Swiss mice (Ezzell *et al.*, 1984; Pezard *et al.*, 1991).

A number of investigations have assessed treatment for disease. The failure of antibiotic prophylaxis against inhalational anthrax has been investigated in mice

(Vančurík, 1966), as has the use of different treatments. Barnes (1947a) investigated the *in vivo* action of penicillin, whilst more recently a number of antibiotics have been investigated for use against *B. anthracis* in a mouse model (Ipatenko *et al.*, 1999).

Mice have been used in the study of anthrax vaccine (Ivins & Welkos, 1988; Percival *et al.*, 1990; Williamson *et al.*, 1990; Coulson *et al.*, 1994; Pezard *et al.*, 1995) and pathogenesis (Welkos *et al.*, 1986; Welkos & Friedlander, 1988; Welkos, 1991). Studies have shown that mice were difficult to protect, using vaccination, against anthrax infection (Welkos & Friedlander, 1988; Welkos *et al.*, 1990). Investigation has shown that toxin negative, capsule positive variants of *B. anthracis* Ames strain retained more virulence than corresponding toxin positive, capsule negative strains in inbred mice (Welkos, 1991). Capsule rather than toxin appears to be the major virulence factor. Thus, vaccines based on PA show reduced efficacy in mice against fully virulent strains (Welkos & Friedlander, 1988).

This chapter describes the work carried out to achieve a new and validated mouse model to test the efficacy of, and immune response to, a vaccine for anthrax.

### **3.1.2 Adjuvants**

Three different adjuvants have been used during the murine investigations. They were a water-in-oil, an aluminium salt adjuvant and a synthetic adjuvant simulating bacterial components. Adjuvants are used to boost the immune response to vaccine antigens (Section 1.8.7). However, water-in-oil emulsions have been used for several decades, the most common being Freund's complete adjuvant (FCA). It comprises

heat-killed *Mycobacterium tuberculosis*, suspended in mineral oil. FCA is one of the most potent adjuvants for stimulating humoral and cell-mediated immunity. However, non-metabolisable mineral oil and mycobacterial antigens can elicit a variety of side effects, including granulomatous reactions, arthritis and experimental allergic encephalomyelitis.

The mineral oil component, without killed mycobacteria, is known as Freund's incomplete adjuvant (FIA). FIA has been used in a number of veterinary preparations, including foot and mouth disease, rabies, and Newcastle disease vaccines. Previously, it was used in experimental human vaccines to enhance responses to influenza and killed poliomyelitis virus. Presently, FIA is not used in widely available human vaccines due to concerns over safety. However, it has been used in experimental vaccines against HIV infection (Birx & Redfield, 1993; Graham & Wright, 1995).

Water-in-oil formulations are useful adjuvants and rely on their ability to target antigen to the immune system. They are efficient for inducing T-cell and antibody responses. The antigen is released from a depot formed at the site of injection (Herbert, 1968).

The second adjuvant used during the murine investigations was alhydrogel (Superfos Biosector, Denmark) an aluminium hydroxide preparation. Vaccine antigens are mixed with aluminium-based adjuvants, such as alhydrogel, to give an adsorbed antigen. The aluminium-based adjuvants are the only adjuvants licensed for human use (Section 1.8.7). They are thought to act by several different mechanisms. Aluminium adjuvants attract eosinophils to the injection site (Walls, 1977) and

activate complement (Ramanathan *et al.*, 1979). However, the majority of evidence indicates the adsorption of antigen to the gel is the key factor in their activity. Mechanisms responsible for absorption include forces such as electrostatic attraction (Al-Shakhshir *et al.*, 1994). Adsorption of antigens to alhydrogel increases efficiency of antigenic presentation to antigen-presenting cells (APC) and upregulates APC activation (Mannhalter *et al.*, 1985).

Alhydrogel also acts as a short-term depot for antigen release. Therefore, antigen is presented in a particulate manner and the rate of antigen targeting is increased (Mannhalter *et al.*, 1985). These properties may explain why alhydrogel works well with small soluble antigens, such as sub-units or toxoids. Immune modulation by alhydrogel affects antibody response. This is characterised by increased production of IL-4 leading to Th<sub>2</sub> stimulation and co-operation in production of antibody (IgG1 dominant in mice; IgG2 in humans; Bomford, 1980).

The third adjuvant, Ribi Trimix, has already been used for the guinea pig model investigations. Details are given in Section 2.3.2.

### **3.13. Recombinant protective antigen**

Recombinant PA (rPA) was a gift from Dr J. Miller (CBD, Porton Down, UK). It was produced as described previously (Miller *et al.*, 1998a). Briefly, rPA, expressed by *B. subtilis* WB600 (pPA101) was precipitated from culture supernatant by ammonium sulphate. Anion exchange chromatography using DEAE STREAMLINE™, to remove media proteins, was followed by anion exchange on an FPLC Mono Q

column to remove rPA and its breakdown products. Contaminating breakdown products were removed using an FPLC Superose 12 gel filtration column, to give 95% pure rPA. The crystal structure of rPA is identical to that of nPA (Miller *et al.*, 1998b).

#### **3.1.4 Enzyme linked immunosorbent assay**

The Enzyme linked immunosorbent assay (ELISA) technique has been valuable in the monitoring and characterisation of immunological responses. ELISAs provide a sensitive and specific method of quantifying antigen or antibody. They combine the specificity of antibodies with the sensitivity of simple spectrophotometric enzyme assays using antigen or antibody conjugated to an easily assayed enzyme. The appropriate antibody or antigen is attached to a solid phase (e.g. disposable microtitre plate) by passive adsorption. Test samples are diluted with buffer containing a wetting agent. The antibody-enzyme conjugate must contain a highly specific antibody coupled to an enzyme with a high turnover number. The coloured product, formed by the action of enzyme on its substrate, may be quantified using a spectrophotometer.

#### **3.1.5 Aim of the experiments**

The primary aim of the series of experiments was to quantify the immune response of mice after vaccination with native and recombinant PA-based vaccine formulations. This was carried out using lymphocyte proliferation assays to provide information on

cellular responses and ELISA to give information on Ig subclasses. The secondary aim was to determine if CMI or humoral immunity was more important for protection in mice after challenge with *B. anthracis* spores.

The investigation initially determined the proliferative response of NIH strain murine lymphocytes to nPA after stimulation *in vitro* for 4, 5 or 7 days. The development of an immune response to nPA was assessed in the second study, using Balb/c mice immunised with purified *B. anthracis* nPA. Lymphocyte proliferation responses were assayed and serum tested by ELISA, to study the development of recall responses and IgG sub-class production after vaccination.

The third investigation, used two different strains of mouse, Balb/c and C57BL6, to determine if immunisation with different rPA vaccine preparations caused the immune response to move to a Th<sub>1</sub> or Th<sub>2</sub> bias. The previous investigation suggested that challenge of SCID/Beige mice reconstituted with lymphocytes and serum from immunised C57BL6 animals would provide information on protection.

Challenge of reconstituted SCID/Beige mice highlighted problems with *B. anthracis* challenge of mice. Study of the effect of *B. anthracis* spore challenge levels in a number of different mouse strains suggested that use of another mouse strain (A/J) would allow successful efficacy studies. A preliminary investigation of A/J strain mice immunised with different vaccine preparations and challenged provided information on protection following immunisation and the lethal challenge levels required for control animals.

The lethal challenge study led to a final investigation of adoptive transfer of



immunity. Donor animals immunised by two vaccination regimen were used for passive transfer of cells and serum to naive animals. Passively immunised animals were challenged to determine whether immune serum or cells were protective.

## **3.2 Method and materials**

### **3.2.1 Experimental animals**

Experimental mice were housed in groups of five to ten animals. All received food and water *ad libitum*. The mice were 6-8 weeks old at the start of the experiment. A number of different mouse strains were used throughout the investigation. Strains and their suppliers were NIH (CAMR, Porton Down, UK), Balb/c (Charles River Laboratories, Margate, Kent. UK), C57BL6 (Charles River Laboratories, Margate, Kent. UK), SCID/Beige (CAMR, Porton Down, UK), CBA (Charles River Laboratories, Margate, Kent. UK), and A/J (Harlan UK Ltd, Oxfordshire, UK).

The initial investigation utilised NIH strain animals. Investigation following the immune response over time used Balb/c inbred animals. To determine the immune response of rPA in different formulations and effect of Th<sub>1</sub> or Th<sub>2</sub> bias, Balb/c and C57BL6 animals were used. Reconstitution of SCID/Beige animals utilised donor cells and serum from C57BL6 animals. Investigation of *B. anthracis* spore challenge used SCID/Beige, Balb/c, C57BL6, CBA and A/J mouse strains.

A/J strain animals were used during the final part of the investigations to elucidate information on which immune components are required to protect mice from *B.*

*anthracis* spore infection.

### 3.2.2 Vaccination regimen

NIH strain mice for the initial investigation were vaccinated with nPA mixed with FIA. nPA in phosphate buffered saline (PBS; 1.0% (w/v) NaCl, 0.075% (w/v) KCl, 0.14% (w/v) Na<sub>2</sub>HPO<sub>4</sub>, and 0.0125% KH<sub>2</sub>PO<sub>4</sub> in water.) was mixed with an equal volume of FIA (Sigma Chemical Co, Poole, UK). The mixture was drawn into a 1 ml glass syringe. A needle with luer locks at either end was used to attach another glass syringe. The mixture was passed backwards and forwards to create a water-in-oil emulsion. A group of six mice was vaccinated with 20 µg nPA (in 100 µl PBS) mixed with 100 µl FIA per mouse by intraperitoneal (i/p) injection. A further six mice received PBS and 100 µl FIA.

Investigation of the immune response over time utilised two different groups. A group of eighteen Balb/c mice was vaccinated at day 0 and 14 with 20 µg nPA and FIA by i/p injection. A further eighteen mice received PBS and FIA as a control group.

Study of the effect of bias to Th<sub>1</sub> or Th<sub>2</sub> used four different groups of mice. A group of 26 Balb/c mice was vaccinated at day 0, 14 and 28 with 2.5 µg rPA and alhydrogel in 50µl by intramuscular (i/m) injection. A further 26 Balb/c mice received Saline and alhydrogel as a vehicle control. A group of 25 C57BL6 mice was vaccinated at day 0, 14 and 28 with 2.5 µg rPA and Ribi Trimix adjuvant in 50µl by i/m injection. A further 25 C57BL6 mice received Saline and Ribi Trimix adjuvant as

a vehicle control.

Reconstitution of SCID/Beige mice utilised a vaccinated group of animals and age-matched unvaccinated controls. C57BL6 mice (30) were immunised with 2.5 µg rPA in Ribi Trimix adjuvant at day 0, 14, 28 and 154 (22).

The investigation of *B. anthracis* spore challenge and different mouse strains used naive unimmunised animals.

The penultimate investigation used three groups of A/J mice. One group (20) received 2.5 µg rPA in Ribi Trimix adjuvant i/m. Another group (20) received 2.5 µg rPA in alhydrogel (25% v/v) i/m. A third control group (21) received saline and alhydrogel (25% v/v) i/m.

The final study involving passive transfer of cells and serum to naive animals used three different donor groups which had been vaccinated as follows: one group (46) received 2.5 µg rPA in Ribi Trimix adjuvant i/m; A second group (46) received 2.5 µg rPA in Alhydrogel (25% v/v) i/m; the third group (31) received saline and Ribi Trimix combined with alhydrogel i/m.

### **3.2.3 Lymphocyte proliferation assay**

In the initial investigation the method described in Section 2.3.3 was used for the proliferation assay. Subsequent experiments used the procedure described below.

### *Initial preparation*

Animals were terminally anaesthetised with a mixture of Ketalar™ (Parke Davis, UK) and Domitor (Smith Kline Beecham Animal Health, UK). Experimental animals were cardiac punctured and a sample of blood obtained. After cervical dislocation, the spleen was removed from the animal and placed in Dulbecco's Modified Eagles Medium (DMEM; Sigma Chemical Co. Poole, UK).

### *Peritoneal wash*

Peritoneal washes were done on syngeneic stock control animals to elicit adherent cells to use as APC's in the T-cell proliferation assay. Animals for peritoneal washes were killed by cervical dislocation. Five ml PBS was passed into the peritoneal cavity. The cavity was washed and washings pooled. The pooled washings were centrifuged and pelleted cells resuspended in serum-DMEM (DMEM with addition of 4% 20 mM L-glutamine, 100 iu/ml Penicillin, 0.1 mg streptomycin/ml and 10% (v/v) foetal calf serum). One hundred microlitres of cell suspension was placed onto a 96-well tissue culture plate. The plate was incubated for 3 hours, at 37°C under CO<sub>2</sub>, to allow macrophages to adhere to the plate. Before use the plates were washed in serum DMEM to remove non-adherent cells.

### *Separation of lymphocytes from spleen*

Spleens from the same treatment groups were pressed through a tea strainer (or cell separator; Falcon, UK) using a syringe plunger to disrupt the spleen. The strainer was washed through with 6 ml serum-free DMEM (DMEM with addition of 4% 20 mM L-glutamine, 100 iu/ml Penicillin, 0.1 mg streptomycin/ml). The cell suspension was centrifuged at 1000rpm for 10 minutes to pellet the cells. The cells were resuspended in serum free DMEM and 3 ml layered onto 4 ml Lymphoprep separation medium (ICN Flow, Thane UK). The cells underwent density centrifugation for 30 minutes at 1000 rpm. A purified band of lymphocytes was collected after centrifugation. The purified cells were washed in 5 ml serum-free DMEM and spun at 500 rpm for 20 minutes. The pellet was then washed in 2 ml serum-free DMEM and recentrifuged at 1000 rpm for 10 minutes.

The packed cells were resuspended in 2 ml serum DMEM. An aliquot (10 $\mu$ l) was taken for viable cell counting using ethidium bromide/acridine orange solution. The solution was prepared by dissolving 50mg Ethidium Bromide and 15mg Acridine Orange in 1 ml 95% ethanol. This solution was made up to 50 ml with water. The stain was diluted 1:100 with PBS for use. The number of viable cells were counted using a fluorescence microscope with a combination of UV and visible light. Viable cells appear green and non viable cells appear orange.

### *Separation using magnetic beads*

T-cells were separated using coated magnetic beads. Dynabeads coated with sheep

anti-mouse IgG (Pan B, M450; Dynal UK) were added in a ratio of 3:1 to mixed B and T-cells. The tubes were incubated on ice for two hours to allow separation to occur. The Dynabeads were removed, using a magnetised rack, and the supernatant washed in serum DMEM. A viable count was done to determine concentration of T-cells

For reconstitution of SCID/Beige mice, magnetic beads were used to separate the lymphocytes into two populations, immune B-cells and immune T-cells. The cells were separated using Dynabeads coated with sheep anti-mouse IgG (Pan B, M450; Dynal UK) to select for B-cells and Dynabeads coated with anti-mouse Pan T marker (anti-Thy1.2; Dynal UK) to select for T-cells. Another sample was prepared containing a mixture of immune B and T-cells. A sample of non immune B- and T-cells was also prepared from control unimmunised animals.

### *Plate preparation*

Doubling dilutions of the relevant specific antigen (or non-specific mitogen) were made in the wells of a microtitre plate pre-coated with APC's, such that 100  $\mu$ l remained per well. Medium alone was used as the negative control. The lymphocyte suspension was aliquoted into each plate well (or into empty cells for crude spleen preparation studies) at a concentration of at least  $10^4$  cells /well (final volume/well 200  $\mu$ l). The plates were incubated for 4 days (37 °C; 5% CO<sub>2</sub>).

### *Addition of $^3\text{H}$ Thymidine*

Twenty four hours before harvesting, 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine in 30  $\mu\text{l}$  Serum DMEM was added to each well. The cells were returned to the incubator for 24 hours. The plates were harvested onto filters. The filters were dried at 60  $^{\circ}\text{C}$  for one hour. The filters were then sealed, scintillant added (Microscint, Packard, Billinghamurst, UK), and counted on a scintillation counter (Topcount; Packard, Billinghamurst, UK).

### *Treatment of results*

Results are given as counts per minute for each well. The results were transformed to give mean  $\pm$  standard error of the mean, for each triplicate, at each antigen (or mitogen) concentration. The stimulation index for the treatment was calculated as before (Section 2.3.3).

The stimulation index (mean  $\pm$  sem) was used to determine if there was a significant difference between antigen (or mitogen) and the corresponding control wells using a Student's t-test.

### **3.2.4 Determination of immunoglobulin sub-classes by ELISA**

A microtitre plate was coated with rPA by adding 100  $\mu\text{l}$  of coating solution (rPA dissolved in PBS at 5  $\mu\text{g/ml}$ ) to each well using a multi-channel pipette. The plate was covered and incubated at 4  $^{\circ}\text{C}$  overnight.

The plate was washed three times with washing/diluent buffer (PBST: 0.05% (v/v) Tween in PBS) using a microplate washer (AM60; Dynex Technologies, Billingham, UK). Blocking buffer (1% (w/v) dried skimmed milk powder in PBST ) was added at 200 µl per well. The plate was incubated at 37 °C for one hour. The plate was washed, as before, and 50 µl blocking buffer added to all wells except for the first well in each row. The initial concentration of serum to be tested was added to the first well in each row (100 µl per well). The samples were mixed by taking the solutions up and down the multi-channel pipette for a minimum of eight times.

After mixing, 50 µl of primary serum dilution was transferred from each of the first wells to the second wells, using the multi-channel pipette and mixed. Solution was transferred from each of the second wells to the third wells, and so on, down to the eleventh column of wells from which 50 µl of solution was discarded. Thus, a two fold dilution series was created in wells 2-11 of each row.

The plate was covered and incubated at 37 °C for 1½ hours. The plate was washed as before. Peroxidase-labelled secondary antibodies (polyclonal goat anti-mouse total Ig, IgG1, IgG2a, IgG2b or IgG3; Harlan Seralab, Kidlington, UK) were used to bind to the mouse anti-PA antibodies. Diluted secondary antibody (diluted for use at a concentration of 1/4000 in 1% Blocking buffer) was added to all wells at 100 µl/well. The plate was covered and incubated at 37 °C for 1 hour, before being washed as before.

Substrate solution was prepared using 100 ml citrate phosphate buffer (pH 4.0; 1 mg/ml), 4 substrate tablets (ABTS; Sigma) and 25 µl hydrogen peroxide solution. The substrate solution (100 µl) was added to every well. The plate was covered and



incubated at 37 °C for 20 minutes before the absorbance was read at 405 nm on a spectrophotometer (Titertek Plus; ICN Flow, UK). The IgG and subclass titre were derived as the reciprocal of the dilution of immune serum giving an optical density of 0.1 units over normal mouse serum. Results were converted to log<sub>10</sub> titre and shown in a graphical or tabular form.

### 3.2.5 Passive transfer or reconstitution with immune components

Investigations involving reconstitution of SCID/Beige mice and passive transfer of immune components to naive A/J animals were carried out by i/p injection of donor cells into recipient animals. SCID/Beige mice received approximately 10<sup>6</sup> cells per animal in 100 µl of DMEM. A/J mice received approximately 10<sup>7</sup> cells per animal in 250 µl of DMEM. Reconstitution and transfer of donor serum used i/p injection to deliver serum to recipient animals. SCID/Beige mice received 200 µl serum per animal. Recipient A/J animals received 500 µl serum per animal. Animals were challenged with *B. anthracis* spores 7 days after receiving cells or 24 hours after receiving serum.

### 3.2.6 *B. anthracis* spore challenge

Two different *B. anthracis* isolates were used for challenge experiments, Ames and STI. Information on the background to Ames has been given in Section 2.3.4. *B. anthracis* STI is an avirulent isolate (pXO1<sup>+</sup>; pXO2<sup>-</sup>) used extensively in the FSU as an attenuated live spore vaccine (Section 1.9.2).

Mice were challenged by i/p injection with a suspension of spores in PBS (Ames challenge  $2 \times 10^3$  cfu in 0.5 ml; STI challenges  $2.5 \times 10^3$ ,  $2.5 \times 10^6$  or  $3.65 \times 10^6$  in 0.1 ml unless otherwise stated). The animals were monitored for the following 14-16 days. Deaths were confirmed due to *B. anthracis* using M'Fadyean staining of blood smears (Turnbull *et al.*, 1993) or isolation on blood agar. Results were presented as the number or percentage surviving after the observation period. The mean time to death was calculated for the reconstituted SCID/Beige and passively immunised A/J challenge groups. The median lethal dose (MLD) was calculated for each strain of mouse receiving *B. anthracis* Ames spores during the lethal challenge investigations using the Reed and Muench method (Reed & Muench, 1938).

### 3.3 Results

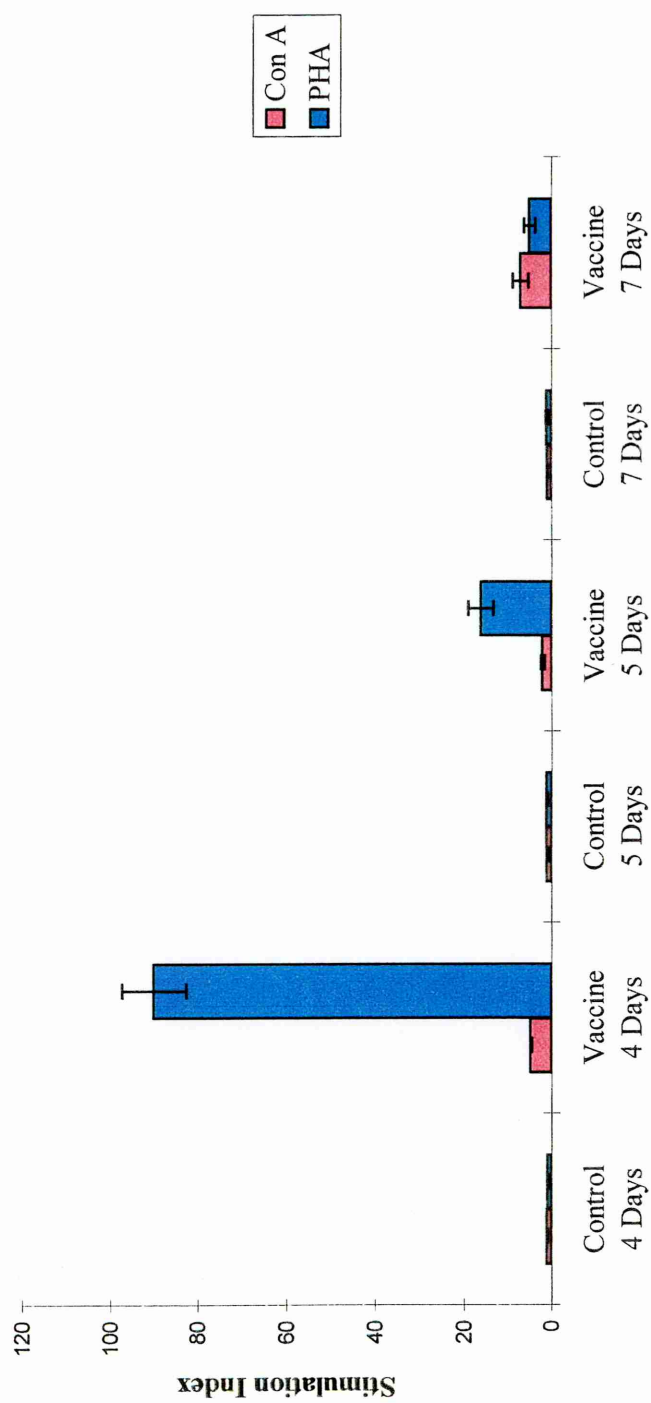
#### 3.3.1 The response of NIH strain murine lymphocytes to native protective antigen

After 4 days, both Con A and PHA cause proliferative responses in the lymphocytes from the vaccinated animals (Figure 3.1). Control lymphocytes do not show any proliferative responses. The responses to PA by both groups are shown in Figure 3.2. No proliferation was seen in any of the control wells. Vaccinated animals showed no responses at 250 and 100  $\mu\text{g/ml}$ . The maximum response was shown at 50 and 25  $\mu\text{g/ml}$ . The response decreases at 10  $\mu\text{g/ml}$ .

Neither lymphocytes from control or vaccinated animal showed any response to Con A after 5 days stimulation (Figure 3.1). PHA stimulated a response in lymphocytes from vaccinated animals, but no response by control lymphocytes. PA, at all levels tested, stimulated proliferation in lymphocytes from vaccinated animals (Figure 3.3). The maximum response was seen at 25  $\mu\text{g/ml}$ . Lymphocytes derived from control animals showed no proliferative response to any of the PA levels tested (Figure 3.3).

Seven days stimulation by either Con A or PHA, caused proliferation in lymphocytes from vaccinated animals (Figure 3.1). Control lymphocytes showed no proliferative responses to either Con A or PHA. Lymphocytes from control and vaccinated groups should have proliferated in response to Con A and PHA. Con A and PHA are non-specific mitogens for lymphocytes. The results suggests that the control lymphocytes were not viable after isolation. After seven days, PA caused proliferation in all lymphocytes from vaccinated animals (Figure 3.4). The response was maximum at 10  $\mu\text{g/ml}$ . Control lymphocytes again showed no proliferative

response to PA (Figure 3.4).



**Figure 3.1** Stimulation indices for different groups after stimulation by mitogens for 4, 5 or 7 days

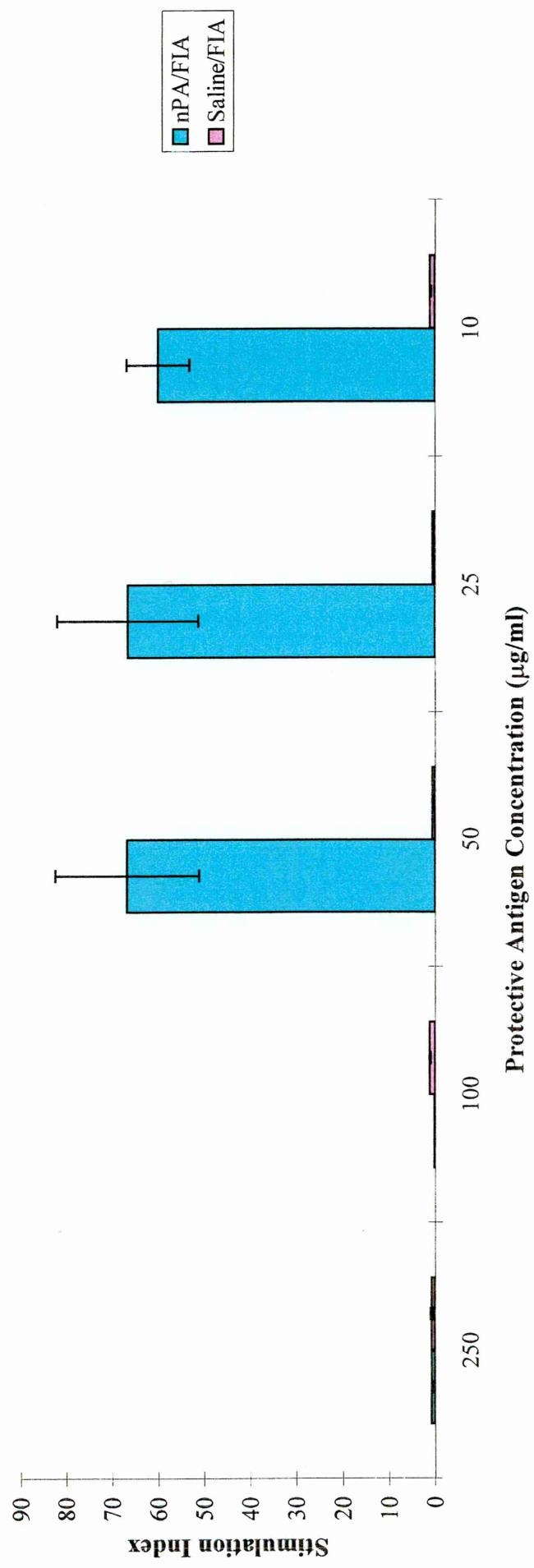
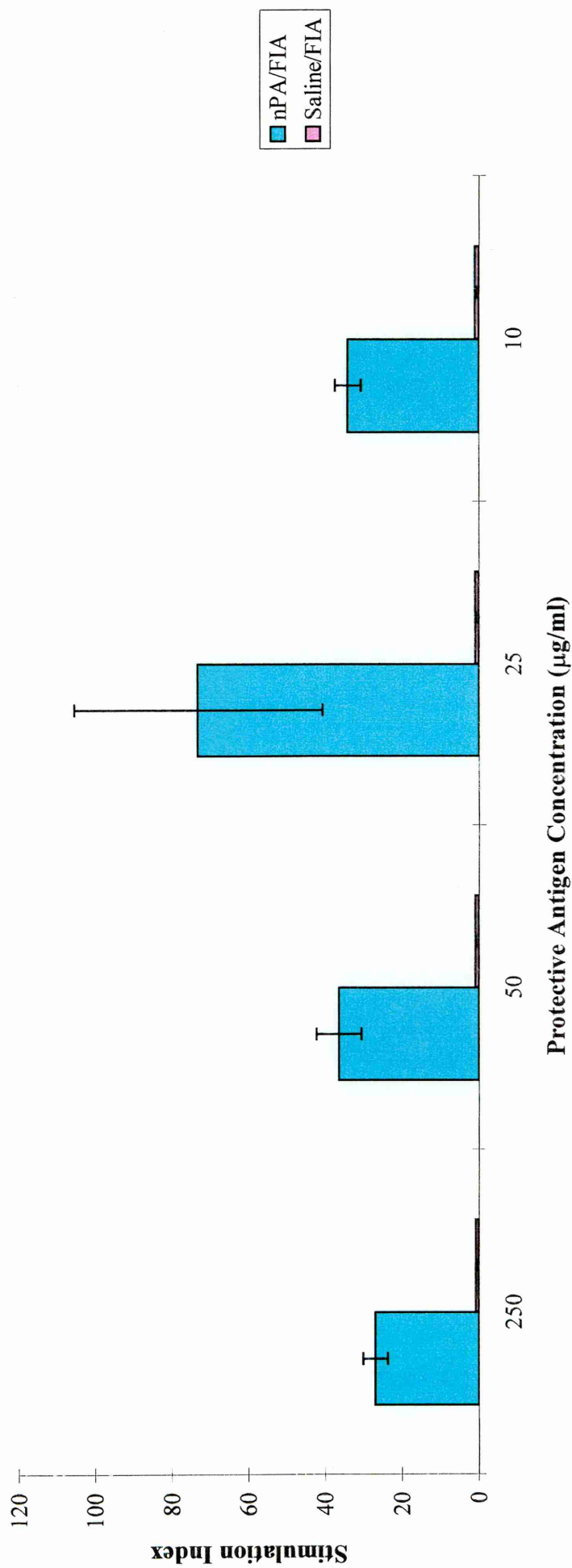


Figure 3.2 PA-specific lymphocyte proliferation after 4 days



**Figure 3.3** PA-specific lymphocyte proliferation after 5 days

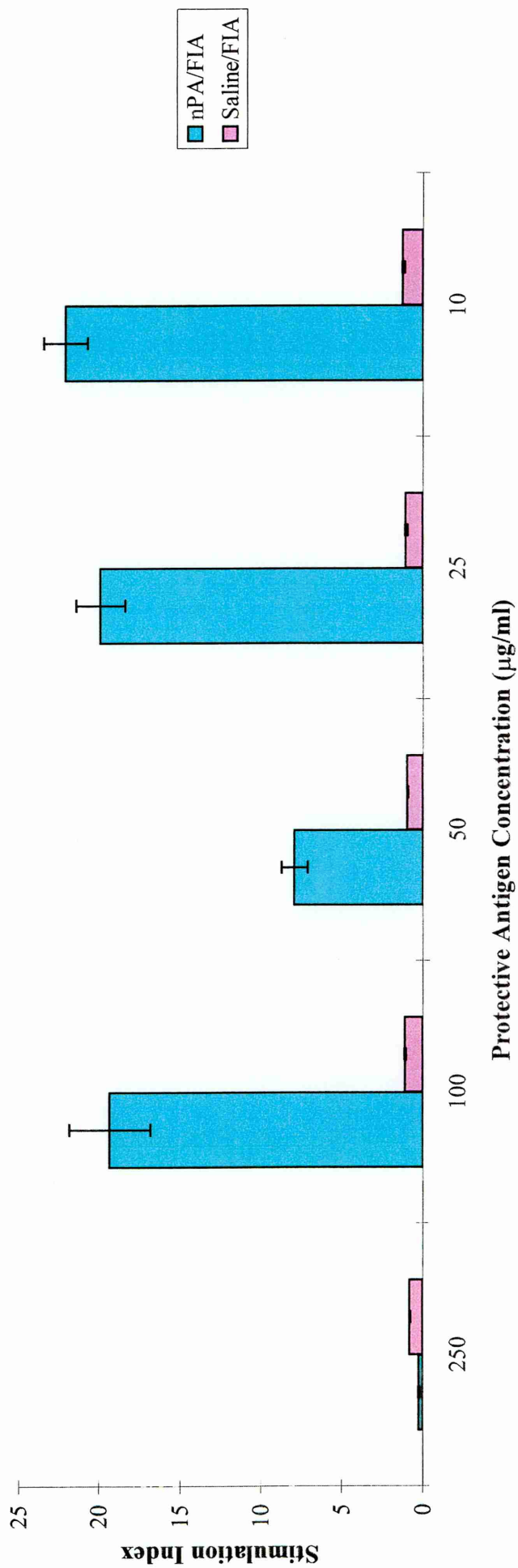


Figure 3.4 PA-specific lymphocyte proliferation after 7 days

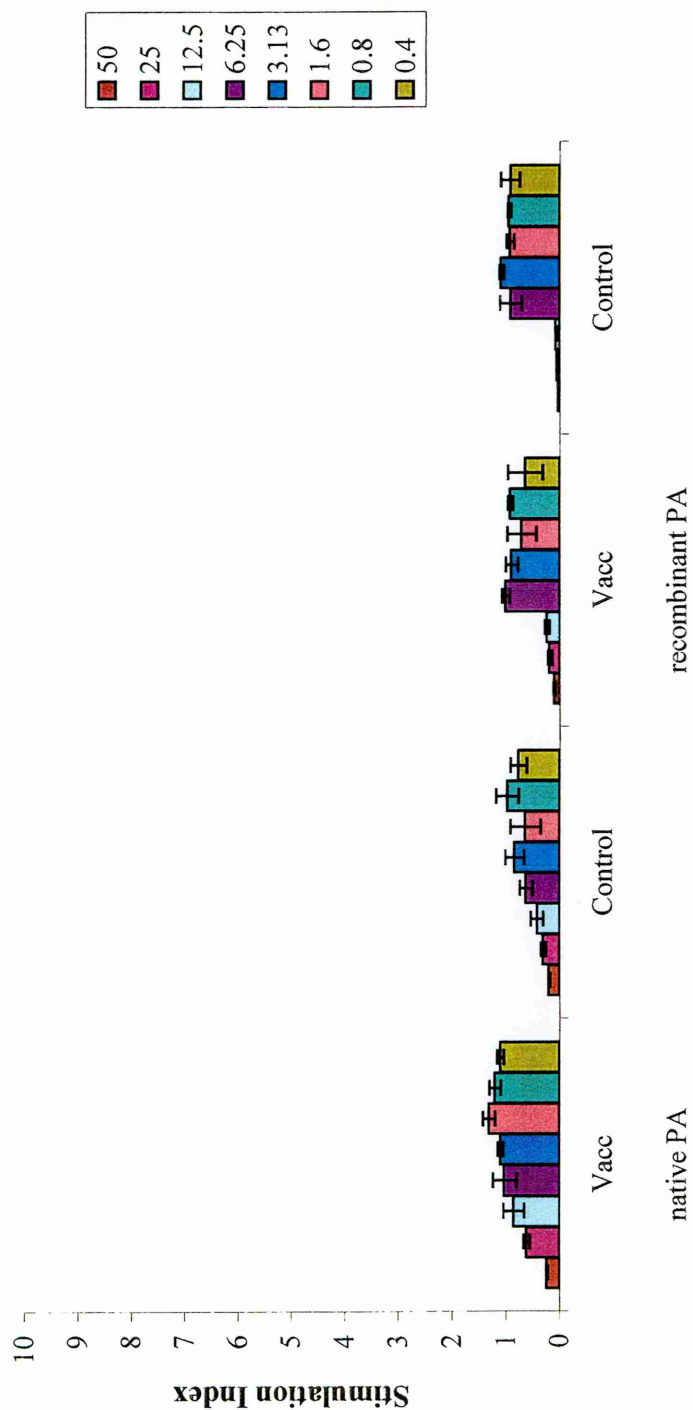


### 3.3.2 The immune response to native and recombinant *Bacillus anthracis* protective antigen, using a Balb/c murine model

#### 3.3.2a Day 30.

During selection of B-cells using Dynabeads (Section 3.2.3) cell counts for the lymphocytes from the vaccinated animals fell from  $2.4 \times 10^6$  to  $1.25 \times 10^6$  cells/ml. The lymphocytes from control animals fell from  $1.65 \times 10^6$  to  $5.50 \times 10^5$  cells/ml.

A lymphocyte proliferation assay, on day 30, showed that lymphocytes derived from both vaccinated and control animals showed no recall response to nPA or rPA (Figure 3.5). The response to the mitogen, Con A, showed that lymphocytes from vaccinated animals (SI 3.42) and, to a certain extent, control animals (SI 2) proliferated. Thus, the isolated lymphocytes were viable and functional. The background readings for untreated cells for the control animals ( $2826.6 \pm 124.3$  cpm) and vaccinated animals ( $2092.36 \pm 59.4$  cpm) was high.



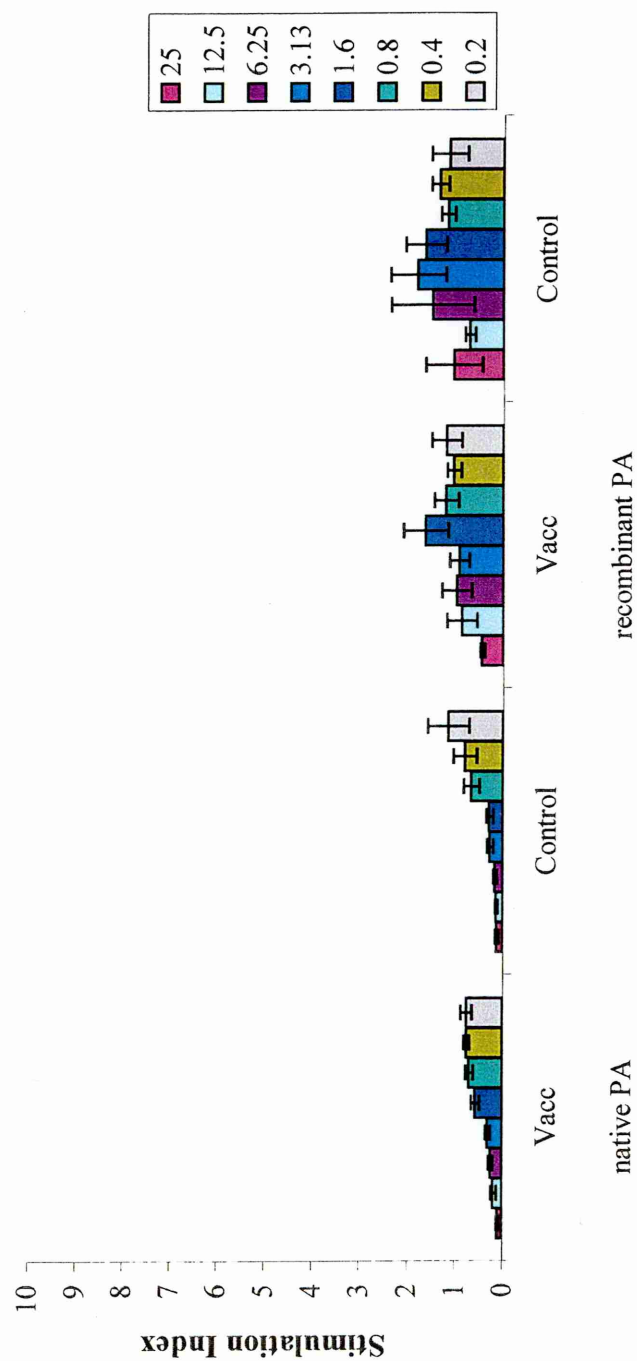
**Figure 3.5** Lymphocyte proliferation responses to different concentrations ( $\mu\text{g/ml}$ ) of native and recombinant PA at day 30

### 3.3.2b Day 35.

Cell counts for the lymphocytes from the vaccinated animals fell from  $1.0 \times 10^7$  to  $7.45 \times 10^6$  cells/ml during selection using Dynabeads (Section 3.2.3) The lymphocytes from control animals fell from  $6.70 \times 10^6$  to  $5.96 \times 10^5$  cells/ml.

At day 35, an assay showed that Con A stimulated a much greater response in both vaccinated and unvaccinated groups. The highest responses by both vaccinated and control animals occurred at  $6.25 \mu\text{g/ml}$ , with SI of 47.77 and 48.61. However, responses to nPA and rPA by all animals showed stimulation indices of less than 2.5 (Figure 3.6). Background counts for vaccinated and control animals were still high ( $2039.76 \pm 345.2$  and  $1636.56 \pm 348.5$  cpm, respectively).

Immunoglobulin isotype levels are shown in Table 3.1. Control animals immunised with FIA and saline showed no PA-specific Ig. The levels for total IgG and IgG1 are similar at  $3.37 \pm 0.31$  and  $3.90 \pm 0.46$ , respectively in animals immunised with FIA and rPA. IgG2a levels are lower at  $2.30 \pm 0.30$ .



**Figure 3.6** Lymphocyte proliferation responses to different concentrations ( $\mu\text{g/ml}$ ) of native and recombinant PA at day 35

Immunoglobulin	FIA/nPA	FIA/saline
Isotype	Balb/c (3 animals)	Balb/c (3 animals)
IgG	$3.37 \pm 0.31$	0
IgG1	$3.90 \pm 0.46$	0
IgG2a	$2.30 \pm 0.30$	0

Note; Value is Log<sub>10</sub> titre of PA specific IgG

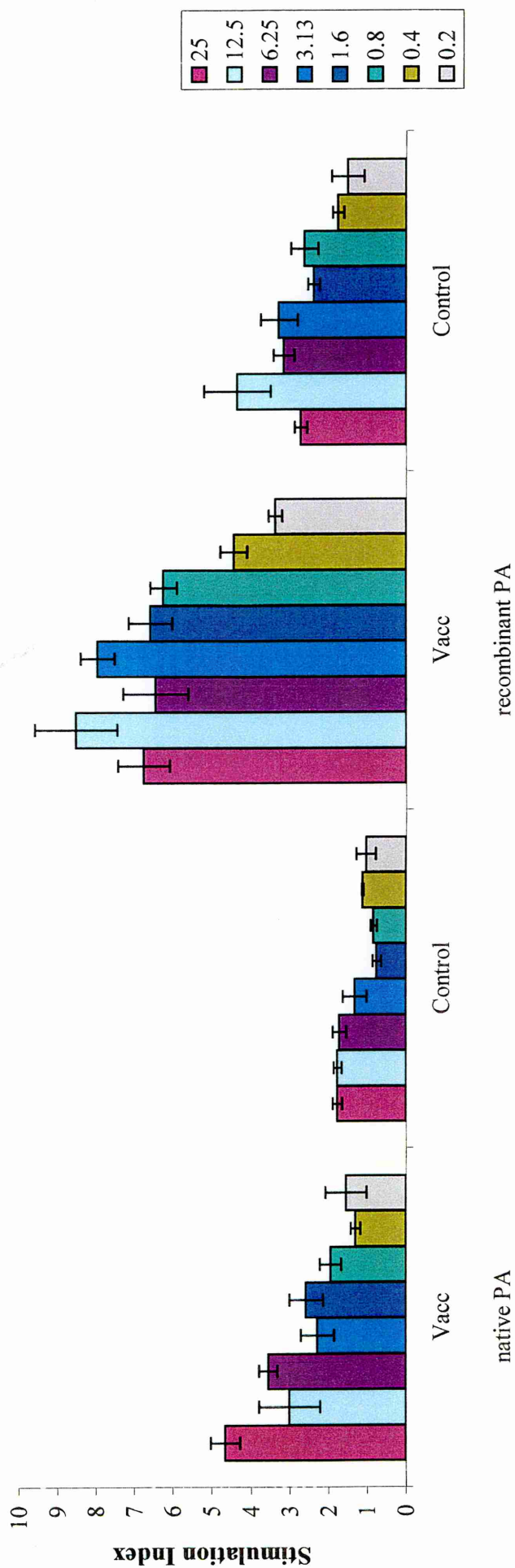
**Table 3.1** Immunoglobulin isotypes detected at day 35 after vaccination.

### 3.3.2c Day 41

During selection of B-cells using Dynabeads (Section 3.2.3) cell counts for the lymphocytes from the vaccinated animals fell from  $1.00 \times 10^7$  to  $5.95 \times 10^6$  cells/ml. The lymphocytes from control animals fell from  $1.30 \times 10^7$  to  $5.35 \times 10^6$  cells/ml.

Con A stimulated responses from both groups of animals (Figure 3.7). The highest responses were  $415.59 \pm 44.11$  (Control) and  $548.92 \pm 26.48$  (nPA vaccinated). Stimulation indices  $>3$  were seen in animals vaccinated with nPA and FIA. Control animals showed a response  $<2$ . Animals, that received nPA, gave a good recall response to rPA. Lymphocytes derived from vaccinated animals showed an SI of  $8.51 \pm 1.06$ , at  $12.5 \mu\text{g/ml}$  rPA. Control animals also showed some proliferation in response to rPA ( $4.34 \pm 0.85$  at  $12.5 \mu\text{g/ml}$ ). This was unexpected because the animals had not received either nPA or rPA. However, the in vitro stimulation shows a significant difference between treated cells and controls. The background wells showed reduced levels, compared to previous assays at days 30 and 35, of  $297.42 \pm 29.9$  cpm (vaccine) and  $381.45 \pm 40.61$  cpm (Control).

Immunoglobulin isotype levels at day 41 are shown in Table 3.2. Control animals immunised with FIA and saline showed no PA-specific Ig. The levels for total IgG and IgG1 were  $3.3 \pm 0.44$  and  $4.16 \pm 0.28$ , respectively in animals immunised with FIA and rPA. The IgG2a level was lower at  $2.15 \pm 0.15$ .



**Figure 3.7** Lymphocyte proliferation responses to different concentrations ( $\mu\text{g/ml}$ ) of native and recombinant PA at day 41

Immunoglobulin	FIA/nPA	FIA/saline
Isotype	Balb/c (3 animals)	Balb/c (3 animals)
IgG	3.30 ± 0.44	0
IgG1	4.16 ± 0.28	0
IgG2a	2.15 ± 0.15	0

Note; Value is Log<sub>10</sub> titre of PA specific IgG

**Table 3.2** Immunoglobulin isotypes detected at day 41 after vaccination.



### 3.3.2d Day 79

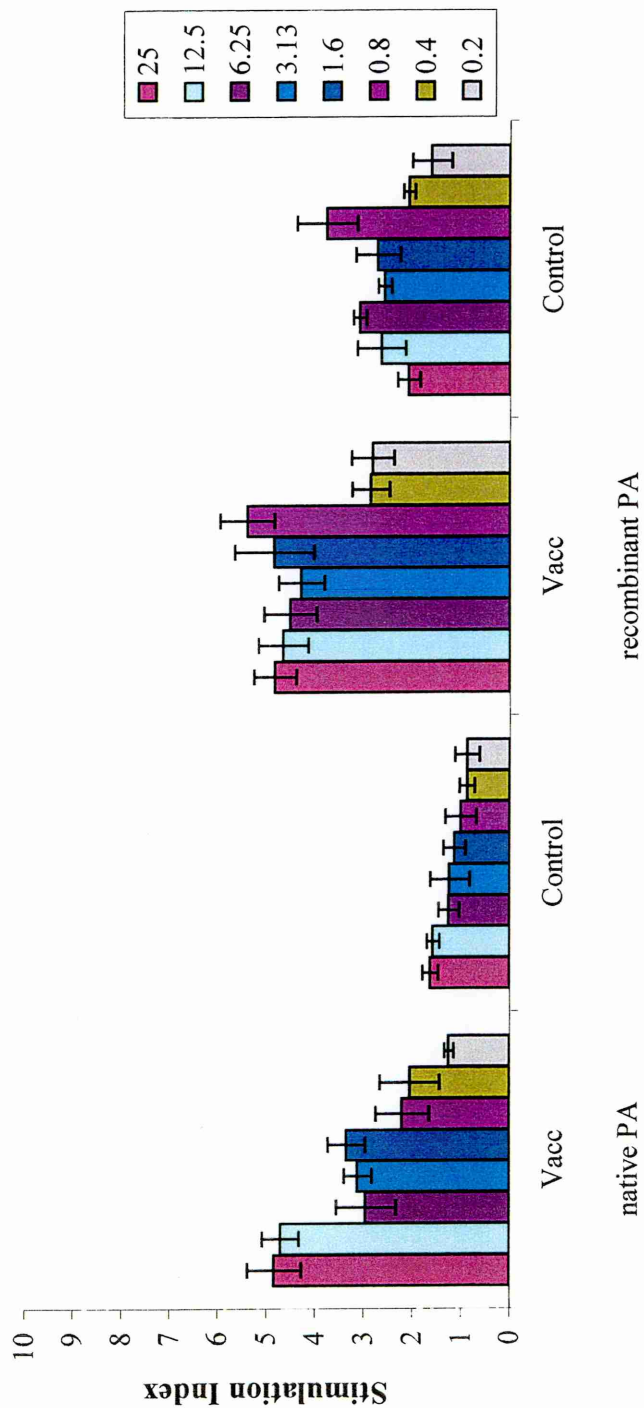
During selection of B-cells using Dynabeads (Section 3.2.3) cell counts for the lymphocytes from the vaccinated animals fell from  $3.35 \times 10^6$  to  $1.95 \times 10^6$  cells/ml. The lymphocytes from control animals fell from  $7.65 \times 10^6$  to  $2.4 \times 10^6$  cells/ml.

At day 79, lymphocytes derived from all groups showed high proliferation responses to Con A. The highest SI's were  $190.49 \pm 30.91$  (nPA Vaccinated) and  $399.39 \pm 69.66$  (Control) at  $3.13 \mu\text{g/ml}$ . These are lower than results seen at day 41 (Figure 3.8). Proliferative responses ( $\text{SI} > 3$ ) to nPA in the vaccinated animals were seen at 25, 12.5, 3.13 and  $1.6 \mu\text{g/ml}$  nPA. There was no proliferation by lymphocytes from control animals to nPA at any level tested.

Vaccinated animals showed proliferative responses to rPA at all levels tested. The highest response was at  $0.8 \mu\text{g/ml}$  rPA with an SI of  $5.39 \pm 0.56$ . Control animals showed a proliferative response at 6.25 and  $0.8 \mu\text{g/ml}$  ( $3.08 \pm 0.13$  and  $3.75 \pm 0.62$ ). The difference between untreated and treated groups are no longer significant at day 79. The background levels were reduced ( $912.96 \pm 95.32$  and  $434.17 \pm 61.80$  cpm for vaccinated and control animals respectively) compared to those levels seen at Days 30 and 35. However, they were slightly higher than those from day 41.

Immunoglobulin isotype levels are shown in Table 3.3. Control animals immunised with FIA and saline showed no PA-specific IgG. The levels for total IgG and IgG1 are  $3.31 \pm 0.20$  and  $4.12 \pm 0.25$ , respectively in animals immunised with FIA and rPA. IgG2a levels are lower at  $2.90 \pm 0.20$ .

Lymphocyte responses appeared to peak at day 41. Earlier responses may be affected by the short period between final boost and assay of the response. Antibody levels elicited by vaccination develop with time. IgG1 appeared to peak at day 41. However, IgG2a peaks at day 79. Total IgG appeared highest at day 35.



**Figure 3.8** Lymphocyte proliferation responses to different concentration (µg/ml) of native and recombinant PA at day 79

Immunoglobulin	FIA/nPA	FIA/Saline
Isotype	Balb/c (6 animals)	Balb/c (6 animals)
IgG	3.31 ± 0.20	0
IgG1	4.12 ± 0.25	0
IgG2a	2.90 ± 0.20	0

Note; Value is Log<sub>10</sub> titre of PA specific IgG

**Table 3.3**      Immunoglobulin isotypes detected at day 79 after vaccination.

### 3.3.3 Murine immune responses to recombinant *Bacillus anthracis* protective antigen.

Investigation of the murine immune response using two different mouse strains, Balb/c and C57BL6, has highlighted some differences between their responses. Vaccinated and control Balb/c animals showed no response to nPA (All SI < 3; Figure 3.9). Vaccinated C57BL6 mice showed good proliferative responses ranging from SI of 7.1 to 14.3. Control C57BL6 animals showed no proliferation.

Re-call responses to rPA by vaccinated C57BL6 animals ranged between SI's of 7 to 12 (Figure 3.10). Control C57BL6 mice showed proliferation responses at 12.5, 3.13, 1.6 and 0.8 µg/ml (SI's of 3.8, 3.1, 3.1 and 3.1). Vaccinated Balb/c animals produced SI's less than 2, signifying no response. Again, control Balb/c animals produced no proliferation (All SI's < 1.4).

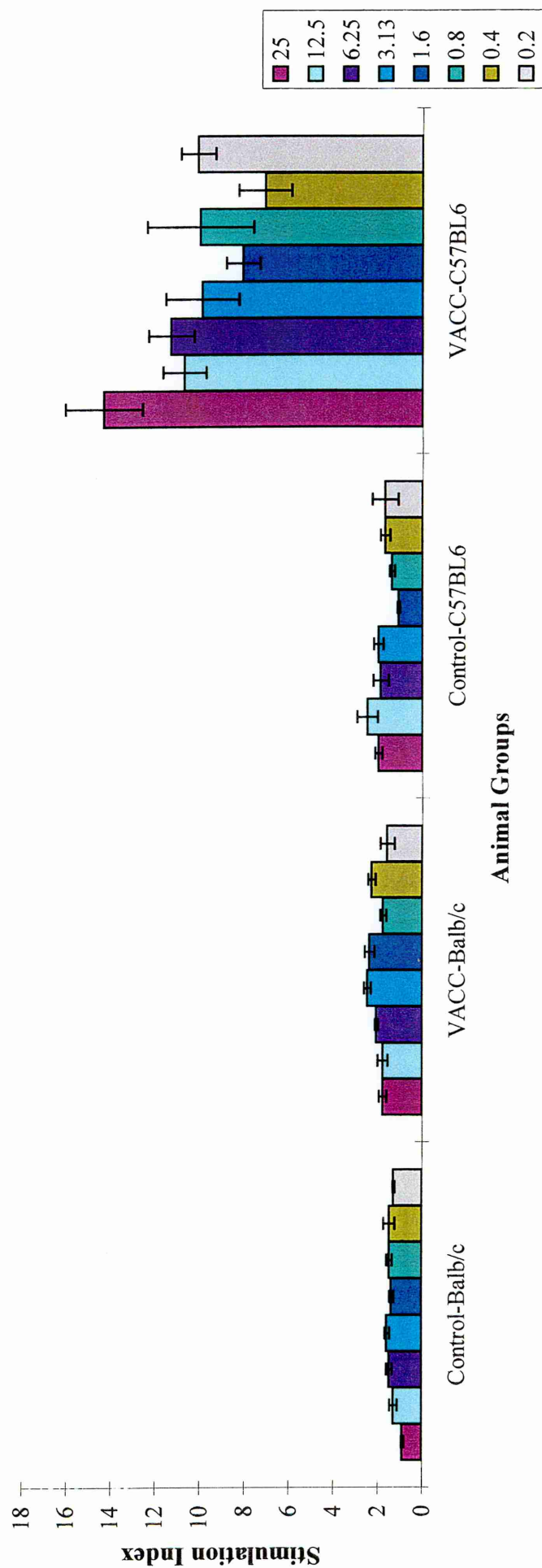
Lymphocytes derived from all groups show proliferation after stimulation by Con A (Figure 3.11). The highest responses were seen in vaccinated (SI  $34.2 \pm 9.5$ ) and control ( $33.3 \pm 8.46$ ) Balb/c animals, at 3.13 µg/ml. However, control C57BL6 animals showed their best response at 1.6 µg/ml (SI  $28.7 \pm 0.99$ ). Whereas vaccinated C57BL6 animals gave the best response at 0.4 µg/ml (SI  $16.7 \pm 0.66$ ). The results show that lymphocytes from all groups are responsive and functional.

The immunoglobulin isotypes (PA specific total IgG, IgG1 and IgG2a) elicited in the two different mouse strains by the different regimen are shown in Figure 3.12. The results showed no PA specific immunoglobulin in either the Balb/c or C57BL6 control groups. Total IgG and IgG1 levels in Balb/c and C57BL6 immunised with,

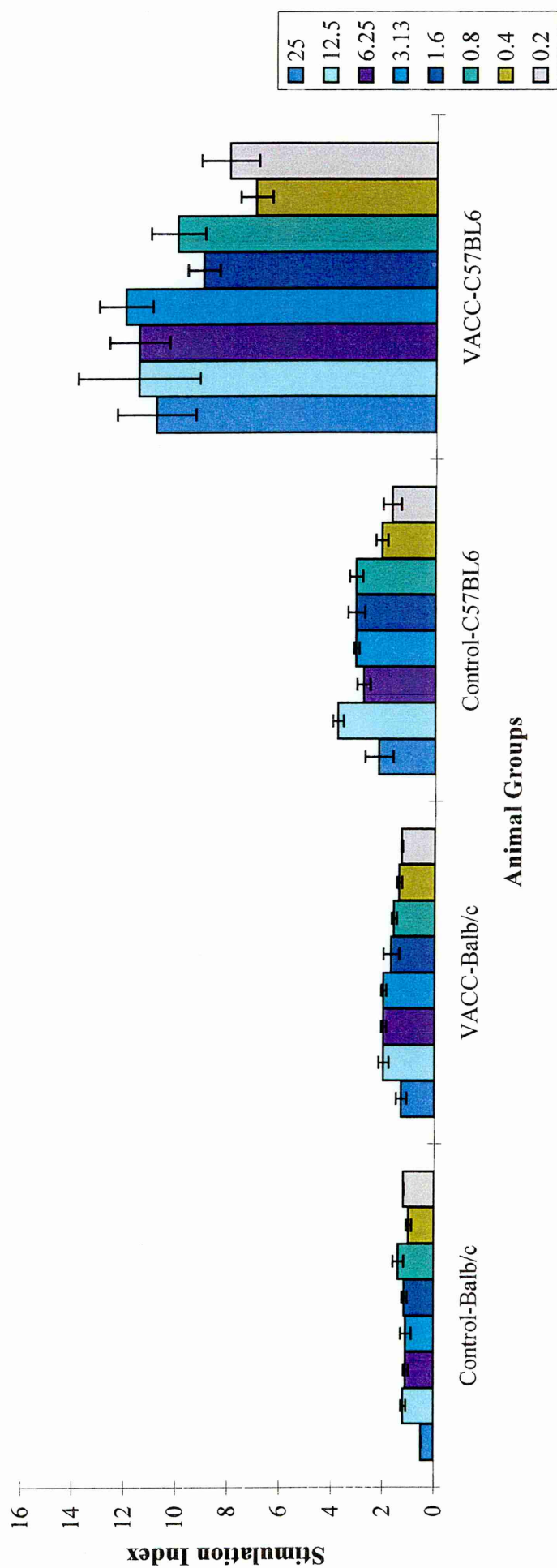
alhydrogel/rPA and Ribi adjuvant/rPA respectively, were the same (4.11 Log<sub>10</sub> titre).

IgG2a levels in the two different mice groups were  $2.98 \pm 0.33$  (Balb/c) and  $3.21 \pm 0.33$  (C57BL6). The differences were not statistically significant by the Student's t-test. IgG2b levels in the two different mice groups were  $3.05 \pm 0.20$  (Balb/c) and 4.11 (C57BL6). The differences were significant by the Student's t-test at  $P < 0.005$  (Degrees of freedom 6).

The results for the Balb/c mice showed a poor lymphocyte response to both nPA and rPA. C57BL6 animals showed a good lymphocyte response to both nPA and rPA. It was expected that the immune systems would be pushed by a combination of vaccination and innate genetic bias to give a Th<sub>1</sub> response in C57BL6 and Th<sub>2</sub> in Balb/c animals. However, the log<sub>10</sub> titres for IgG isotypes and the IgG2a/IgG1 ratios suggest that both strains of mice were showing a Th<sub>2</sub> response. The results suggest that PA induces a Th<sub>2</sub> response that is not dependent on the adjuvants used in the vaccine preparation in this study.

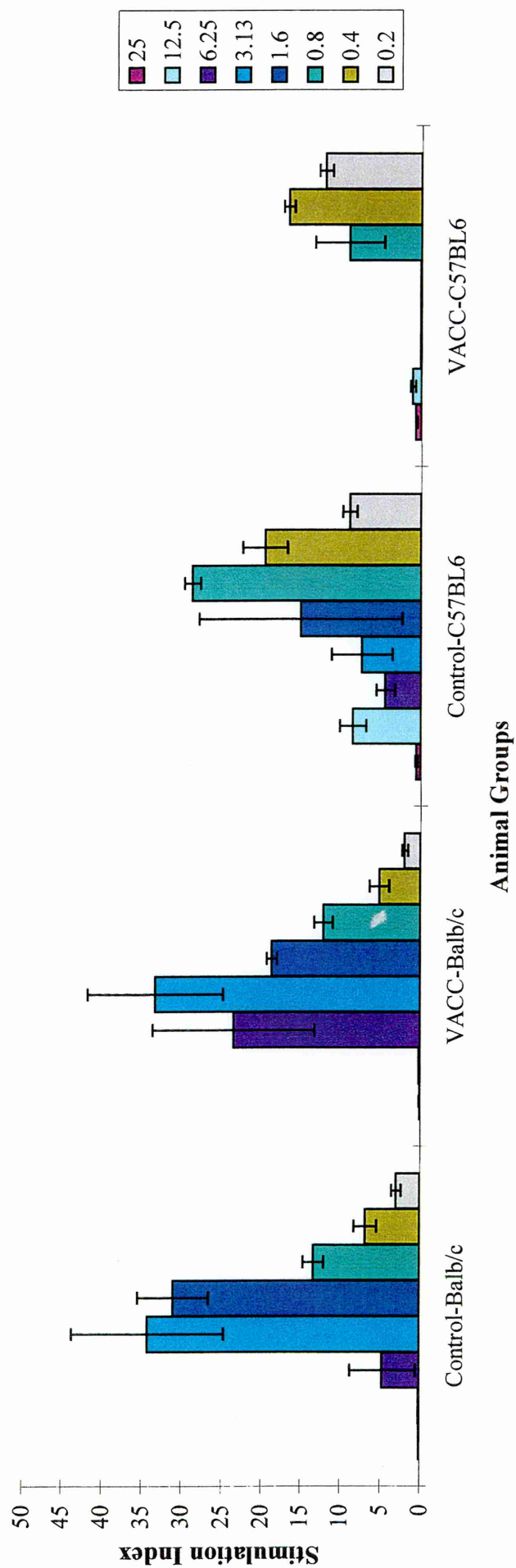


**Figure 3.9** Lymphocyte proliferation responses to different concentrations ( $\mu\text{g/ml}$ ) of native PA

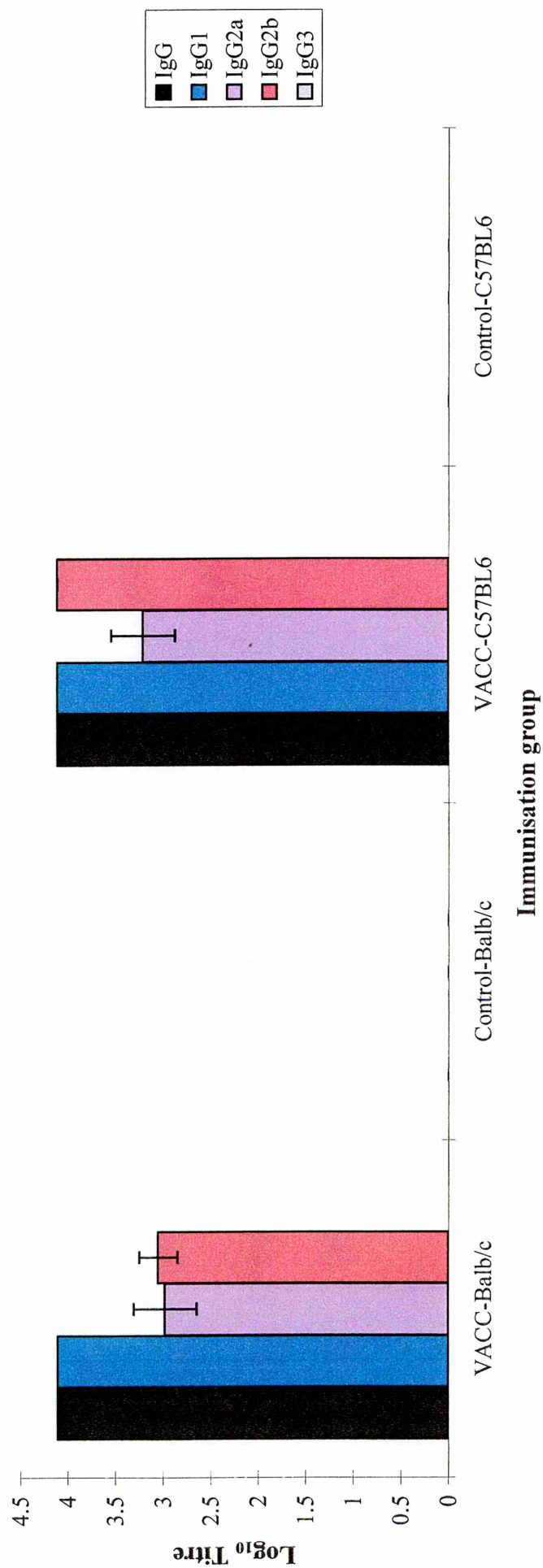


**Figure 3.10** Lymphocyte proliferation response to different concentrations ( $\mu\text{g/ml}$ ) of recombinant PA





**Figure 3.11** Lymphocyte proliferation responses to different concentrations (µg/ml) of Concanavalin A



**Figure 3.12** Immunoglobulin isotypes elicited by vaccination in different strains of mice

### 3.3.4 Reconstitution of SCID/Beige mice with immune cells and serum.

SCID/Beige animals, from the immune B-cell group, received  $4.4 \times 10^6$  cells. Animals in the immune T-cell group received  $2.5 \times 10^6$  cells. The group reconstituted with a mixture of B and T-cells received a total of  $2.9 \times 10^6$  cells, composed of  $1.9 \times 10^6$  B-cells and  $1 \times 10^6$  T-cells. The non-immune B and T-cell group received  $2.2 \times 10^6$  cells. The immunoglobulin isotypes of the serum, in terms of PA-specific  $\log_{10}$  titre, are shown in Table 3.4. The mice were challenged by the i/p route using *B. anthracis* Ames ( $2 \times 10^3$  cfu ).

The time to death was calculated for each experimental group (Table 3.5). The Student's t-test showed that there are no significant differences between immune serum and non-immune serum groups or between immune B and T-cells and non-immune B and T-cells. This may be due to controls surviving the challenge. Although there is no statistical significance, groups receiving either immune serum, immune T-cells or immune B and T-cells survived longer than the non-immune serum group.

Immunoglobulin Isotype	Ribi/rPA	Ribi/saline
IgG	5.16 ± 0.09	0
IgG1	5.16 ± 0.09	0
IgG2a	4.01 ± 0.1	0

Note; Value is Log<sub>10</sub> titre of PA specific IgG

**Table 3.4** Immunoglobulin isotypes of pooled immune and non-immune C57BL6 serum used for reconstitution of SCID/Beige mice.

Group	No. of Survivors/Total No. (%)	Time to Death (Days)
Immune B Cells	2/6 (33)	11 ± 1.8
Immune T-Cells	3/6 (50)	10.5 ± 2.5
Immune B + T-Cells	2/5 (40)	9.8 ± 2.8
Immune Serum	3/6 (50)	9.2 ± 3.1
Non-Immune B + T-Cells	1/3 (33)	11 ± 4.0
Non-immune Serum	1/3 (33)	8.3 ± 3.8

Note: Values are means ± standard errors of the means. Animals were challenged by i/p injection using *B. anthracis* Ames ( $2 \times 10^3$  cfu).

**Table 3.5** Protection of reconstituted SCID/Beige mice against challenge with *B. anthracis* Ames

### 3.3.5 Investigation of *B. anthracis* spore challenge in different mouse strains

#### 3.3.5a Challenge of SCID/Beige, CBA, Balb/c and C57BL6 mice with *B. anthracis*

##### Ames spores

Table 3.6 shows the survival of four different mouse strains after challenge with *B. anthracis* spores following 15 days observation. All strains had MLD values that are <20 cfu of *B. anthracis* Ames strain. The results were surprising after the survival of the SCID/Beige animals in the previous study. The investigation suggested that all four strains are susceptible to challenge with fully virulent *B. anthracis* spores. The tendency for spores to clump together may lead to difficulty in accomplishing a challenge to which all the control animals succumb, leaving surviving immunised animals the chance to show the efficacy of the vaccination regimen. Reports in the literature suggested that challenge of A/J mice with non-capsulated *B. anthracis* spores would provide an experimental model (Welkos et al., 1986).

Mouse Strain	Challenge Level (cfu)			
	$1.30 \times 10^4$	$1.60 \times 10^3$	$2.25 \times 10^2$	$2.00 \times 10^1$
SCID/Beige	0/10	0/10	0/10	0/10
Balb/c	0/10	0/10	1/10	3/10
C57BL6	0/10	0/10	0/10	0/10
CBA	0/10	0/10	0/10	0/10

Note: Animals were challenged with *B. anthracis* Ames spores.

**Table 3.6**      Survival of different mouse strains after *B. anthracis* spore challenge

### 3.3.5b Challenge of A/J and Balb/c animals with *B. anthracis* STI spores

Results in terms of survivors and MLD for both, A/J and Balb/c strain mice, after challenge with toxigenic, non-capsulated STI strain spores are given in Table 3.7. The results showed that Balb/c animals are relatively resistant to infection with STI. A/J mice appear to be susceptible to STI infection in a dose-dependent manner. These results agreed with reports in the literature that the A/J mouse was less resistant to challenge with non-capsulated strains of *B. anthracis* than Balb/c mice (Welkos *et al.*, 1986; 1990). The dose-dependent response suggested that A/J animals could be used to study which components of the immune system were important in protection against *B. anthracis* infection.



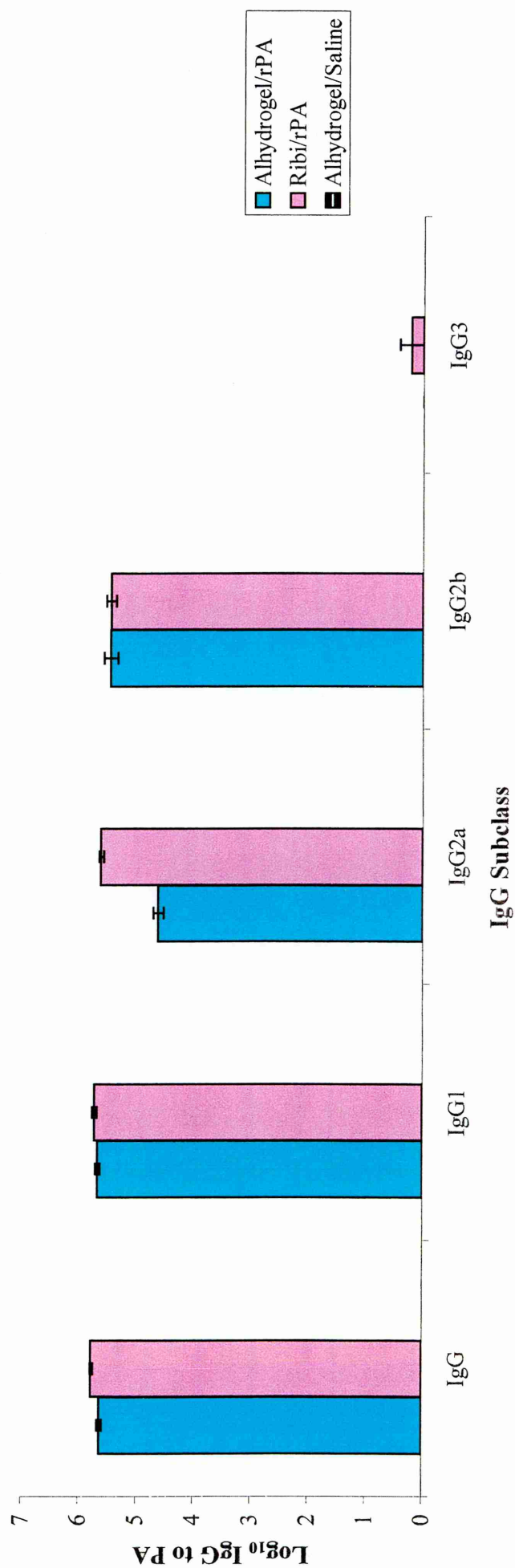
Mouse Strain	Challenge Level (cfu)	Survivors	MLD (cfu)
A/J	$9.00 \times 10^4$	0/5	1349.5
	$1.15 \times 10^4$	3/5	
	$1.80 \times 10^3$	1/5	
	$2.10 \times 10^2$	4/5	
	$8.70 \times 10^1$	5/5	
	$6.50 \times 10^1$	5/5	
Balb/c	$3.00 \times 10^8$	0/5	Between $1.25 \times 10^7$ and $3.00 \times 10^8$
	$1.25 \times 10^7$	5/5	
	$1.75 \times 10^6$	5/5	
	$9.00 \times 10^4$	5/5	
	$1.80 \times 10^3$	5/5	

**Table 3.7**      Susceptibility of two different mouse strains to *B. anthracis* STI spore challenge

### 3.3.6 Investigation of protection by two different vaccine preparations using A/J mice

After vaccination, PA specific Ig isotypes were determined by ELISA in the three groups of animals. A summary of the IgG isotypes elicited by the different vaccine preparations are shown in Figure 3.13. Control mice immunised with alhydrogel and saline showed no PA-specific Ig. Mice from the group immunised with alhydrogel and rPA showed PA-specific total IgG, IgG1, IgG2a and IgG2b. However, there was no detectable response for PA-specific IgG3. Mice receiving Ribi and rPA during immunisation showed PA-specific response to all isotypes of Ig. The responses by both groups were very similar. IgG2a showed a difference between the two groups ( $4.621 \pm 0.09$  and  $5.616 \pm 0.041$  Log<sub>10</sub> titre, for alhydrogel/rPA and Ribi/rPA respectively). The difference was significant according to a Student's t-test ( $P < 0.5\%$ ). IgG3 was found in one Ribi/rPA immunised animal leading to a mean ( $\pm$  sem) of  $0.211 \pm 0.211$  log<sub>10</sub> titre.

The results from the challenge experiments are shown in Table 3.8. A proportion of the control animals (immunised with alhydrogel/saline) receiving the low dose challenge survived (60%). All animals in the alhydrogel/rPA and Ribi/rPA groups survived challenge at the low level. All control animals at the high challenge dose died within 5 days. Animals from the alhydrogel/rPA and Ribi/rPA groups survived the 14 day observation period after challenge.



**Figure 3.13** Immunoglobulin isotypes induced by vaccine preparations in A/J mice

Challenge Dose (cfu spores)	Vaccine Preparation	Survivors/No. challenged (%)
Low ( $2.5 \times 10^3$ )	Alhydrogel and saline	6/10 (60)
	Alhydrogel and rPA	10/10 (100)
	Ribi Trimix and rPA	10/10 (100)
High ( $3.65 \times 10^6$ )	Alhydrogel and saline	0/11(0)
	Alhydrogel and rPA	10/10 (100)
	Ribi Trimix and rPA	10/10 (100)

Note: Animals were challenged with *B. anthracis* STI.

**Table 3.8**      Survival of A/J mice after vaccination and challenge with two different levels of *B. anthracis*

### **3.3.7 Transfer of immune and non immune serum and cells into naive animals**

Cell suspensions isolated from vaccinated animals were used to immunise passively naive A/J strain mice. Animals in the Ribi:alhydrogel:saline group received  $5.75 \times 10^7$  cells. The Ribi/rPA animals were given  $4.13 \times 10^6$  cells. Animals immunised with alhydrogel and rPA received  $6.25 \times 10^7$  cells.

Results, in terms of time to death, for the different experimental groups were calculated (Table 3.9) and compared using a Student's t-test. Animals receiving alhydrogel/rPA serum survived significantly better than animals receiving control serum ( $P < 0.5\%$ ). Likewise, animals from the Ribi/rPA serum group survived longer than control serum animals ( $P < 0.5\%$ ). There was no significant difference between the alhydrogel/rPA and Ribi/rPA animals, as expected.

The passive transfer of alhydrogel/rPA or Ribi/rPA cells to mice did not significantly increase their survival compared to mice receiving control cells. Thus, the survival of animals receiving alhydrogel/rPA cells did not vary significantly compared with animals receiving Ribi/rPA cells.

Group	No. survivors/total No. (%)	Time to death (Days)
rPA/Alhydrogel Serum	8/9 (88)	13.8 ± 0.2
rPA/Ribi Serum	8/9 (88)	13.0 ± 0.9
Alhydrogel/Ribi/Saline Serum	0/8 (0)	3.3 ± 0.5
rPA/Alhydrogel Cells	1/9 (11)	2.9 ± 0.3
rPA/Ribi Cells	0/9 (0)	2.9 ± 0.5
Alhydrogel/Ribi/Saline Cells	0/6 (0)	4.0 ± 0.5

Note: Animals were challenged with  $2.5 \times 10^6$  cfu *B. anthracis* STI; Values are means ± standard errors of the means

**Table 3.9**      Protection of passively immunised A/J mice after challenge with *B. anthracis* STI spores

### 3.4 Discussion

The present study has provided data on the immune response to native and recombinant PA and on which immune components are important for protection in a murine challenge model. Preliminary investigation of the murine response to nPA, using NIH mice, showed that PA-specific proliferative responses could be elicited by immunisation. Thus, vaccination with nPA and FIA leads to a memory response, a prerequisite of any vaccine preparation.

Study of the immune responses with time has shown that antibodies and lymphocyte recall responses develop following immunisation with nPA/FIA. Peak lymphocyte responses are seen at day 41. Earlier responses may have been effected by the period of time between final boost and assay of the response. The investigation has shown that functional assays can detect PA-specific antibody and cellular responses after vaccination. The responses are vital to successful recognition of antigen by the host immune system.

Study of vaccination in two different mouse strains showed PA-specific responses occur in both groups. However, vaccination combined with genetic bias of the mouse strains did not lead to the expected outcomes. It was thought that a Th<sub>2</sub> response would appear in Balb/c animals and a Th<sub>1</sub> response in C57BL6 animals. From the data gained, it is clear that PA induces a Th<sub>2</sub> response that was independent of the adjuvants or genetic background used in the immunisation regimen.

Reconstitution of SCID/Beige mice by adoptive transfer of immune components

from immunised C57BL6 animals followed by challenge with fully virulent *B. anthracis* spores was problematic because of initial difficulties in selecting a mouse model that performed consistently against challenge with *B. anthracis*. However, the results suggested that immune serum, T-cells or B and T-cells were required for increased survival. The failure of challenge levels to kill all control animals led to investigation of different *B. anthracis* strains and susceptibility of various mouse strains to infection.

Data produced from challenge with fully virulent *B. anthracis* showed that all four mouse strains, SCID/Beige, Balb/c, CBA and C57BL6, were highly susceptible to infection (MLD<20cfu). Challenge of Balb/c and A/J mice with the live vaccine non-capsulated strain, STI, agreed with reports in the literature (Welkos *et al.*, 1986, 1990). Balb/c mice were resistant to challenge, whilst A/J mice were susceptible and died in a dose-dependent manner. Thus, A/J mice were identified as a valid mouse model and used as the model for challenge of animals immunised by direct vaccination or adoptive transfer of immune components.

Investigation of protection using A/J mice immunised by two different regimen showed that PA elicited a high antibody response independent of the immunising preparation. This agreed with earlier data from the combined C57BL6 and Balb/c study. Challenge of actively-immunised A/J mice led to information on the dose required to kill all control animals. Data from the challenge demonstrated that antibody was important for survival. Alhydrogel is known to stimulate good antibody responses whilst Ribi adjuvant containing MPL, TDM and CWS, is known to stimulate good antibody responses and increased cellular responses (Rudbach *et al.*, 1995; Lövgren-Bengtsson, 1998).



Passive transfer of specific immunity is a principal technique for analysing the immune response providing information on which immune components are sufficient in surviving challenge. Passive transfer of immune or non-immune serum and cells into naive A/J mice suggested that PA-specific antibody was the key to survival against challenge in the experimental A/J mouse model. However, immune cells may not have been successful in increasing survival because their effects were overwhelmed by the speed of onset of disease following challenge. It would be expected that B and T-cell co-operation would be an advantage in overcoming a lethal challenge. PA is a T-cell dependent antigen (Williamson *et al.*, 1990) and memory responses generated by vaccination would allow T-cells to assist B-cells in recognition and response to challenge.

## CHAPTER 4.0

### Conclusions

## 4.0 Conclusions

The work reported in this thesis has examined the immune response to PA in two species, guinea pig and mouse towards the identification of immune correlates of protection. The guinea pig has not proven a reliable model in this report, but work comparing different strains of mice has identified a valid murine model.

Use of immunological techniques has provided more data on immunity to *B. anthracis* infection. Examination of guinea pigs proliferative responses could not be correlated with aerosol challenge results. DTH and lymphocyte proliferation responses have shown that recall responses can be generated by immunisation. However, high variation occurred between animals within the same groups. This could be due to a combination of variation within the lymphocyte assay and the outbred nature of the animal strain used. Thus, it was difficult to use the guinea pig model to demonstrate a memory response which is a requisite of any useful immunising preparation.

Investigation of the murine immune responses to immunisation with rPA preparations has suggested that antibody is the key mediator of protection against *B. anthracis* challenge, although B and T-cell co-operation is expected to be essential in establishing the immune response.

Both guinea pig and murine investigation have highlighted differences that occur between different species. Although many species are available for immunological and infection studies with *B. anthracis* (Section 1.8.9) they rely on the animal's immune system to generate protective responses. Guinea pigs are partially protected

against parenteral and inhalational challenge by alum-precipitated human chemical vaccine (Ivins & Welkos, 1988; Turnbull *et al.*, 1990b; Ivins *et al.*, 1995; Jones *et al.*, 1996a). Immunisation with native or recombinant PA with Ribi adjuvant, but not with alhydrogel, yields strong protection (McBride *et al.*, 1998; Jones *et al.*, 1996a). Survival of rabbits after immunisation with alum-precipitated human chemical vaccine has been reported to correlate with the humoral immune response and protection (Pitt *et al.*, 1998). Rhesus macaques are reported to be protected against challenge after immunisation with alum-precipitated human chemical vaccine (Darlow *et al.*, 1956; Pitt *et al.*, 1996; Ivins *et al.*, 1998;). Immunisation with alum-containing chemical vaccine is known to provide protection for approximately two years (Ivins *et al.*, 1996; Darlow *et al.*, 1956).

The aim of this study was to achieve results on an immune correlate of protection. This would allow a method of determining an individual's immunological status in terms of protection against *B. anthracis* infection. The investigation has been successful in this aim, by demonstrating that in a valid mouse model of infection that antibody is the key mediator of protection against anthrax infection. Further work would be required to elucidate the roles of antibody and cellular responses in human disease. However, this would be difficult due to the nature of the disease.

Investigation of the pathogenesis of *B. anthracis* by both the parenteral and inhalation routes of infection may provide new sites of intervention in terms of treatment and vaccination against *B. anthracis* infection. In order to cause disease a bacterial pathogen must: adhere to host tissues; invade host tissues; multiply in host tissues; evade host innate and acquired immunity and cause damage. During these steps a range of antigens may be expressed *in vivo* before toxin release, thus

allowing the design and development of increasingly efficacious vaccines (Hanna & Ireland, 1999). Techniques, such as laser confocal microscopy, fluorescent associated cell sorting, cytokine markers and *in vitro* assays may provide beneficial data (Guidi-Rontani, *et al.*, 1999). Further investigation of passive transfer of naive mice may allow a definitive answer as to how immune effectors play a role in protection against *B. anthracis* infection.

In conclusion, the investigations contained within this report have added to knowledge about the mechanism of protection against *B. anthracis* infections. The results will provide further data for the continued development of a second-generation anthrax vaccine.

## References

Abalakin, V.A. & Cherkasskii, B.L. (1978). Use of inbred mice as a model for the indication and differentiation of *Bacillus-anthraxis* strains. *Zhurnal. Mikrobiol. Epidemiol. Immunobiol.*, 146-147.

Abdenour, D., Larouze, B., Dalichaouche, M. & Aouati, M. (1987). Familial occurrence of anthrax in Eastern Algeria. *J. Infect. Dis.* 155, 1083-1084.

Advisory Committee on Dangerous Pathogens (1995). *Categorisation of biological agents according to hazard and categories of containment*. HSE Books. Fourth Edition.

Albrink, W.S., Brooks, S.M., Biron, R.E. & Kopel, M. (1960). Human inhalation anthrax: a report of three fatal cases. *Am. J. Pathol.*, 36, 457-471.

Aleksandrov, N.I., Gefen, N.E., Garin, N.S., Gapochko, K.G., Sergeev, V.M., Smirnov, M.S., Tamarin, A.L. & Shlyakhov, E.N. (1959). Experiment of mass aerogenic vaccination of people against anthrax. *Voyenno-Meditsinskii Zhurnal*, 8, 27-32.

Aleksandrov, N.I., Gefen, N.E., Budak, A.P., Ezepchuk, LuV, V., Filippenko, A.I. & Runova, V.F. (1960). Utilization of effective chemical vaccines against certain zoonoses. I. Production of chemical depot anthrax vaccine and determination of its effectiveness in animal experiments. *Zhurnal. Mikrobiol. Epidemiol. Immunobiol.*, 32, 42. Cited by Hambleton *et al.*, 1984.

Alexeyev, O.A., Morozov, V.G., Suzdaltseva, T.V., Mishukov, A.S. & Steinberg,

L.A. (1994). Impaired neutrophil function in the cutaneous form of anthrax. *Infection*, 22, 281-282.

Al-Shakhshir, R., Regnier, F., White, J.L. & Hem, S.L. (1994). Effect of protein adsorption on the surface charge characteristics of aluminium-containing adjuvants. *Vaccine*, 12, 472-474.

Anon (1983). Epidemic was one of Africa's worst. *Harare Herald*.

Anon (1991). Middle east war heightens biological warfare preparedness. *ASM News*, 57, 116-118.

Appleton, J.L.T. (1925). *Bacterial Infection with special reference to dental practice*. Lea & Febiger, Philadelphia and New York. 140.

Auerbach, S. & Wright, G.G. (1955). Studies on immunity in anthrax. VI. Immunizing activity of protective antigen against various strains of *Bacillus anthracis*. *J. Immunol.*, 75, 129-133.

Bail, O. (1904a). *Zbl. Bakt.* 1., 36, 266. Cited by Sterne (1959).

Bail, O. (1904b). *Zbl. Bakt.* 1., 37, 270. Cited by Sterne (1959).

Barnes, J.M. (1947a). Penicillin and *B. anthracis*. *J. Pathol. Bacteriol.*, 59, 113-115.

Barnes, J.M. (1947b). The development of anthrax following the administration of



spores by inhalation. *Brit. J. Exp.Path.*, 28, 385-394.

Beall, F.A., Taylor, M.J. & Thorne, C.B. (1962). Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. *J. Bact.*, 83, 1274-1280.

Beall F.A. & Dalldorf, F.G. (1966). The pathogenesis of the lethal effect of anthrax toxin in the rat. *J. Inf. Dis.*, 116, 377-389.

Bell, J.H. (1880). On anthrax and anthracemia in woolsorters, heifers and sheep. *BMJ*, 2, 656-657.

Belton, F.C. & Strange, R.E. (1954). Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*: medium and methods of production. *Br. J. Exp. Pathol.*, 35, 144-152.

Benson, E.L., Huynh, P.D., Finkelstein, A. & Collier, R.J. (1998). Identification of residues lining the anthrax protective antigen channel. *Biochemistry*, 37, 3941-3948.

Birx, D.L. & Redfield, R.R. (1993). Immunotherapeutic strategies in the treatment of HIV infection and AIDS. *Curr. Top. Immunol.*, 5, 600-607.

Böhm, R. (1990). Resistance, survival, sterilisation and disinfection of spores of *Bacillus anthracis*. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 99-101.

Boismenu, R. & Havran, W.L. (1997). An innate view of  $\gamma\delta$  T cells. *Curr. Opinion. Immunol.*, 9, 57-63.

Bomford, R. (1980). The comparative selectivity of adjuvants for humoral and cell mediated immunity. I. Effect on the antibody response to bovine serum albumin and sheep red blood cells of freunds incomplete and complete adjuvant, alhydrogel, *Corynebacterium parvum*, *Bordetella pertussis*, Muramyldipeptide and saponin. *Clin. Exp. Immunol.*, 39, 426-434.

Boor, A.K. (1955). An antigen prepared *in vitro* effective for immunisation against anthrax. I. Preparation and evaluation of the crude protective antigen. *J. Infect. Dis.*, 97, 194 - 202.

Boor, A.K. & Tresselt, H.B. (1955a). An antigen prepared *in vitro* effective for immunisation against anthrax. II. Concentration of the protective antigen by chemical fractionation. *J. Infect. Dis.*, 97, 203 - 206.

Boor, A.K. & Tresselt, H.B. (1955b). Protection of sheep against anthrax by immunization with an antigen prepared *in vitro*. *Am. J. Vet. Res.*, 16, 425-428.

Brachmann, P.S., Gold, H., Plotkin, S.A., Fekety, R., Werrin, M. & Ingraham, N.R. (1962). Field evaluation of a human anthrax vaccine. *Am. J. Pub. Health*, 52, 632 - 645.

Bradarić, N. & Punda-Polić, V. (1992). Cutaneous anthrax due to penicillin-resistant *Bacillus anthracis* transmitted by an insect bite. *The Lancet*, 340, 306-307.

Broster, M.G. & Hibbs, S.E. (1990). Protective efficacy of anthrax vaccines against aerosol challenge. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 91-92.

Buchner, H. (1888). *Arch. F. Hyg.*, 8, 217. Cited by Young *et al.*, 1946.

Carman, J.A., Hambleton, P. & Melling, J. (1985). *Bacillus anthracis*. In *Isolation and Identification of Microorganisms of medical and veterinary importance*. Edited by C.H. Collins & G.M. Grange Society of Applied Bacteriology Technical Services No. 21, Academic Press, London, 207-214.

Cartwright, M.E., McChesney, A.E. & Jones, R.L. (1987). Vaccination-related anthrax in three llamas, *J. Amer. Vet. Med. Assoc.*, 191, 715 - 716.

Christie, A.B. (1987). Anthrax. In *Infectious Diseases: Epidemiology and Clinical Practice*. Edited by A.B. Christie. 4th Edn, Churchill Livingstone, Edinburgh, 983-1003.

Christopher, G.W., Cieslak, T.J., Pavlin, J.A. & Eitzen, E.M. (1997). Biological warfare a historical perspective. *J. A. M. A.*, 278, 412-417.

Clarke, V.L. & Bavoil, P.M. (1994). Bacterial pathogenesis. Part A. Identification of virulence factors. *Meth. in Enzym.*, 235, 29-140.

Cole, L.A. (1996). The specter of biological weapons. *Scientific American*,

Coulson, N.M., Fulop, M. & Titball, R.W. (1994). Effect of different plasmids on colonisation of mouse tissues by the aromatic amino acid dependent *Salmonella typhimurium* SL3261. *Microb. Pathog.*, 16, 305-311.

Darlow, H.M., Belton, F.C. & Henderson, D.W. (1956). The use of anthrax antigen to immunise man and monkey. *The Lancet*, 476-479.

de Boer, A.S. & Diderichsen, B. (1991). On the safety of *Bacillus subtilis* and *B. amyloiquefaciens*: a review. *Appl. Micro. Biotechnol.*, 36, 1-4.

De Vos, V. (1990). The ecology of anthrax in the Kruger National Park, South Africa. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 19-23.

Dirckx, J.H. (1981). Virgil on anthrax. *Am. J. Derm.path.*, 3, 191-195.

Dong, S. L. (1990). Progress in the control and research of anthrax in China. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 104-105.

Dongzheng, Y. (1998). Anthrax and anthrax researches in China. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 4.

Druett, H.A., Henderson, D.W., Packman, L. & Peacock, S. (1953). Studies on respiratory infection. I. The influence of particle size on respiratory infection with anthrax spores. *J. Hyg (Cambs)* ., 51, 359-371.

Druett, H.A. (1969). A mobile form of the Henderson apparatus. *J. Hyg.(Camb)*., 67, 437-448.

Ebedes, H. (1981). A new look at anthrax. In *Wildlife diseases of the Pacific basin and other countries. Proceedings of the 4<sup>th</sup> international conference of the wildlife disease association.* (25-28 August). Australia. Edited by M.E. Fowler. 85-88.

Elkin, B.T., Gates, G.C., Ellsworth, T. & Dragon, D.C. (1998). Meso- and macro-ecology of anthrax in bison in northern Canada: the 1993 Mackenzie outbreak as a case study. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 6.

Eurich, F.W. & Hewlett, R.T. (1930). *Bacillus anthracis*. In *A System of Bacteriology in relation to Medicine*. Volume V. Overall Editors P. Fildes. & J.C.G. Ledingham. Volume Editors F.C. Minett. S.L. Cummins. & R.T. Hewlett. HMSO, Chapter X, 437-478.

Ezzell, J.W., Ivins, B.E. & Leppla, S.H. (1984). Immuno-electrophoretic analysis, toxicity and kinetics of *in vitro* production of the protective antigen and lethal factor components of *Bacillus anthracis* toxin. *Infect. Immun.*, 45, 761-767.

Ezzell, J.W., Mikesell, P., Ivins, B.E. & Leppla, S.H. (1985). The genetic basis of

Pasteur's attenuation of *Bacillus anthracis* cultures. In *The World's Debt to Pasteur*.

Edited by H. Koprowski. Wistar Symposium Series Vol 3. Alan R. Liss, Inc. 111.

Fairbrother, R.W. (1962). *A Text-Book of Bacteriology*. William Heinemann Medical Books Ltd, London. 353-357.

Fellows, P.F. (1996). A survey of worldwide strains of *Bacillus anthracis*. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 31-33.

Fellows, P.F., Linscott, M.K. & Ivins, B.E. (1998). Anthrax vaccine efficacy against *Bacillus anthracis* strains of diverse geographical origin. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, P12, 48.

Fouet, A., Sirard, J-C. & Mock, M. (1996). Virulence gene determinants. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, , 87, 84-85.

Fouet, A., Mesange, S., Tosi-Couture, E., Gounon, P. & Mock, M. (1998). *Bacillus anthracis* S-Layer. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 16.

Friedlander, A.M. (1986). Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J. Biol.Chem.*, 261, 7123-7126.

Friedlander, A.M., Welkos, S.L., Pitt, M.L.M., Ezzell, J.W., Worsham, P.L., Rose, K.J., Ivins, B.E., Lowe, J.R., Howe, G.B., Mikesell, P. & Lawrence, W.B. (1993a). Postexposure prophylaxis against inhalation anthrax. *J. Infect. Dis.*, 167, 1239-1242.

Friedlander, A. M., Bhatnagar, R., Leppla, S.H., Johnson, L. & Singh, Y. (1993b). Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. *Infect. Immun.*, 61, 245-252.

Gladstone, G.P. (1946). Immunity to anthrax. Protective antigen present in cell-free culture filtrates. *Br. J. Exp. Pathol.*, 27, 394-418.

Gladstone, G.P. (1948). Immunity to anthrax. Production of the cell-free protective antigen in cellophane sacs. *Br. J. Exp. Pathol.*, 29, 379-389 .

Glenny, A.T., Pope, C.G., Waddington, H. & Wallace, U. (1926). The antigenic value of toxoid precipitated by potassium alum. *J. Pathol. Bacteriol.*, 29, 38.

Gold, H. (1967). Treatment of anthrax. *Fed. Proc.*, 26, 1563-1568.

Gordon, V.M., Klimpel, K.R., Arora, N., Henderson, M.A. & Leppla, S.H. (1995). Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. *Infect. Immun.*, 63, 82 - 87.

Graham, B.S. & Wright, P.F. (1995). Candidate AIDS vaccines. *Drug Therapy*, 333, 1331-1339.

Green, B.D., Battisti, L., Koehler, T.M., Thorne, C.B. & Ivins, B.E. (1985). Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infect. Immun.*, 49, 291-297.

Green, M., Rogers, D., Russell, P., Stagg, A.J., Bell, D.L., Eley, S.M., Titball, R.W. & Williamson, E.D. (1999). The SCID/Beige mouse as a model to investigate protection against *Yersinia pestis*. *FEMS Immun. Med. Microbiol.*, 23, 107-113.

Greenfield, W.S. (1880). Preliminary note on some points in the pathology of anthrax with especial reference to the modification of the properties of the *Bacillus anthracis* by cultivation, and to the protective influence of inoculation with a modified virus. *Proc. Royal Society, London*, 30, 557-560.

Guidi-Rontani, C., Duflot, E. & Mock, M. (1997). Anthrax lethal toxin-induced mitogenic response of human T-cells. *FEMS Microbiol. Lett.*, 157, 285-289.

Guidi-Rontani, C., Weber-Levy, M., Labruyere, E. & Mock, M. (1999). Germination of *Bacillus anthracis* spores within alveolar macrophages. *Mol. Microbiol.*, 31, 9-17.

Haines, B.W., Klein, F. & Lincoln, R.E. (1965). Quantitative assay for crude anthrax toxins. *J. Bacteriol.*, 89, 74-83.

Hambleton, P., Carman, J. A. & Melling, J. (1984). Anthrax: the disease in relation to vaccines. *Vaccine*, 2, 125-32.



- Hanna, P.C., Acosta, D. & Collier, R.J. (1993). On the role of macrophages in anthrax. *Proc. Natl. Acad. Sci. USA.*, 90, 10198-10201.
- Hanna, P.C., Kruskal, B.A., Ezekowitz, R.A.B., Bloom, B.R. & Collier, R.J. (1994). Role of macrophage oxidative burst in the action of anthrax lethal toxin. *Mol. Med.*, 1, 7-18.
- Hanna, P.C. (1998). Anthrax pathogenesis and host response. *Curr. Top. Microbiol. Immun.*, 225, 13-35.
- Hanna, P.C. & Ireland, J.A.W. (1999). Understanding *Bacillus anthracis* pathogenesis. *Trends. Microbiol.*, 7, 180-182.
- Henderson, D.W. (1952). An apparatus for the study of airborne infection. *J. Hyg (Camb)*, 50, 53-68.
- Henderson, D.W., Peacock, S. & Belton, F.C. (1956). Observations on the prophylaxis of experimental pulmonary anthrax in the monkey. *J. Hygiene (Cams)*, 54, 28-36.
- Herbert, W.J. (1968). The mode of action of mineral-oil emulsion adjuvants on antibody production in mice. *Immunology*, 14, 301-318.
- Hoover, D.L., Friedlander, A.M., Rogers, L.C., Yoon, I.-K., Warren, R.L. & Cross, A.S. (1994). Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor alpha and interleukin-6 by increasing

intracellular cyclic AMP. *Infect. Immun.*, 62, 4432-4439.

Hugh-Jones, M.E. & Hussaini, S.N. (1974). An outbreak of anthrax in Berkshire. *Vet. Record*. 94, 228-232.

Hugh-Jones, M. (1996). World Situation 1993/4. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 1-2.

Hugh-Jones, M. (1998a). Anthrax, human & livestock - Kazakhstan (Kustani). *Program for Monitoring Emerging Disease*. 17 August.

Hugh-Jones, M. (1998b). Anthrax, human - Central Asian Republics. *Program for Monitoring Emerging Disease*. 27 October.

Hugh-Jones, M. (1998c). Anthrax - USA (North Dakota). *Program for Monitoring Emerging Disease*. 28 July.

Hugh-Jones, M. (1999). World occurrence of anthrax 97. Internet Site [Http://www.vetmed.lsu.edu/whocc/mp\\_world.htm](http://www.vetmed.lsu.edu/whocc/mp_world.htm). July 1999.

Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Friedlander, A.M., Hauer, J., McDade, J., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K. & Tonat, K. (1999). Anthrax as a biological weapon; medical and public health management. *J.A.M.A*, 281, 1735-1745.

Ipatenko, N.G., Yakovleva, T.N. & Bakhtarov, S.I. (1999). Treatment of animals suffering from anthrax. *Moscow Veterinariya*, 4, 18-23. Abstract only.

Ivins, B.E., Ezzell, J.W., Jemski, J., Hedlund, K.W., Ristroph, J.D. & Leppla, S.H. (1986). Immunization studies with attenuated strains of *Bacillus anthracis*. *Infect. Immun.*, 52, 454-458.

Ivins, B.E. & Welkos, S.L. (1988). Recent advances in the development of an improved human anthrax vaccine. *Eur. J. Epidemiol.*, 4, 12-19.

Ivins, B.E., Welkos, S.L., Knudson, G.B. & Little, S.F. (1990). Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. *Infect. Immun.*, 58, 303-308.

Ivins, B.E., Welkos, S.L., Little, S.F., Crumrine, M.H. & Nelson, G.O. (1992). Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. *Infect. Immun.*, 60, 662-668.

Ivins, B.E., Fellows, P.F. & Nelson, G.O. (1994). Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* spore challenge in guinea pigs. *Vaccine*, 12, 872-874.

Ivins, B., Fellows, P., Pitt, L., Estep, J., Farchaus, J., Friedlander, A. & Gibbs, P. (1995). Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against aerosol *Bacillus anthracis* spore challenge in guinea pigs.

*Vaccine*, 13, 1779-1784.

Ivins, B.E., Fellows, P.F., Pitt, M.L.M., Estep, J.E., Welkos, S.L., Worsham, P.L. & Friedlander, A.M. (1996). Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* aerosol spore challenge in Rhesus monkeys. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 125-126.

Ivins, B.E., Pitt, M.L.M., Fellows, P.F., Farchaus, J.W., Benner, G.E., Waag, D.M., Little, S.F., Anderson Jr, G.W., Gibbs, P.H. & Friedlander, A.M. (1998). Comparative efficacy of experimental vaccine candidates against inhalation anthrax in rhesus macaques. *Vaccine*, 16, 1141-1148.

Jacotot, H. & Virat, B. (1954). La longevite des spores de *B. anthracis* (premier vaccin de Pasteur). *Ann. Inst. Pasteur*, 87, 215-217.

Jones, M.N., Beedham, R.J., Turnbull, P.C.B., Fitzgeorge, R.B. & Manchee, R.J. (1996a). Efficacy of the UK human vaccine against aerosol challenge of virulent *Bacillus anthracis* spores, tested using a guinea pig model. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin Special Supplement*. 87, 123-124.

Jones, M.N., Beedham, R.J., Turnbull, P.C.B. & Manchee, R.J. (1996b). Antibiotic prophylaxis for inhalational anthrax. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin Special Supplement*. 87, 127-128.

Kaufmann, S.H.E. & Ledel, C.H. (1994). Application of knockout mice to the experimental analysis of infections with bacteria and protozoa. *Trends. Micro.*, 2, 235-241.

Keppie, J., Harris-Smith, P.W. & Smith, H. (1963). The chemical basis of the virulence of *Bacillus anthracis* IX: its aggressins and their mode of action. *Br. J. Exp. Pathol.*, 44, 446-453.

Klemm, D.M. & Klemm, W.R. (1959). A history of Anthrax. *J. Am.Vet.Med.Assoc.*, 458-462.

Klimpel, K.R., Arora, N. & Leppla, S.H. (1994). Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. *Mol. Microbiol.*, 13, 1093-1100.

Knudson, G.B. (1986). Treatment of anthrax in man: history and current concepts. *Mil. Med*, 151, 71-77.

Kobuch, W.E., Davis, J., Fleischer, K., Isaacson, M. & Turnbull, P.C.B. (1990). A clinical and epidemiological study of 621 patients with anthrax in western Zimbabwe. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 34-38.

Kochi, S.K., Schiavo, G., Mock, M. & Montecucco, C. (1994). Zinc content of the *Bacillus anthracis* lethal factor. *FEMS Microbiol. Lett.*, 124, 343-348.

Koch, R.H. (1876). The aetiology of anthrax based on the ontogeny of the anthrax bacillus. Reprinted in *Medical Classics* (1938), 2, 745-820.

Ladel, C.H., Blum, C., Dreher, A., Reifenberg, K. & Kaufmann, S.H.E. (1995). Protective role of  $\gamma/\delta$  T cells and  $\alpha/\beta$  T cells in tuberculosis. *Eur. J. Immunol.*, 25, 2877-2881.

Lalitha, M.K., Anandi, V., Walter, N.M., John, L., Devadatta, J.O., Pulimood, B.M. & Koshi, G. (1990). Unusual forms of anthrax - a clinical problem. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 38-40.

Legge, T.M. (1934). *Industrial Maladies*. Humphrey Milford, London. 36-44. Cited by Fairbrother, R.W. (1962). 354.

Leppla, S.H. (1982). Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. *Proc. Natl. Acad. Sci. USA*, 79, 3162 - 3166.

Leppla, S.H., Ivins, B.E. & Ezzell, J.W. (1985). Anthrax toxin. *Microbiology - 1985*. American Society for Microbiology, Washington, DC. 63-66.

Leppla, S.H., Friedlander, A.M. & Cora, E.M. (1988). Proteolytic activation of anthrax toxin bound to cellular receptors. *Bacterial Protein Toxins*, Gustav Fischer, Stuttgart, 111 - 112.

Lightfoot, N.F., Scott, R.J.D. & Turnbull, P.C.B. (1990). Antimicrobial susceptibility of *Bacillus anthracis*. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 95-98.

Lincoln, R.E., Klein, F., Walker, J.S., Haines, B.W., Jones, W.I., Mahlandt, B.G. & Friedman, R.H. (1965). Successful treatment of Rhesus monkeys for septicemic anthrax. *Antimicrob. Agents Chemo.*, 759-763.

Lincoln, R. E., Walker, J. S., Klein, F., Rosenwald, A. J. & Jones, W. I., Jr. (1967). Value of field data for extrapolation in anthrax. *Fed. Proc.*, 26, 1558-1562.

Lincoln, R.E. & Fish, D.C. (1970). Anthrax toxin. In *Microbial Toxins III*, 1st Edition. Edited by T.C. Montie, S. Kadis. & S.J. Ajl. Academic Press, New York. Chapter 9, 361-414.

Little, S.F. & Knudson, G.B. (1986). Comparative efficacy of *Bacillus anthracis* live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. *Infect. Immun.*, 52, 509-512.

Little, S.F., Novak, J.M., Lowe, J.R., Leppla, S.H., Singh, Y., Klimpel, K.R., Lidgerding, B.C. & Friedlander, A.M. (1996). Characterisation of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. *Microbiology*, 142, 707-715.

Little, S.F., Ivins, B.E., Fellows, P.F. & Friedlander, A.M. (1997). Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea

pigs. *Infect. Immun.*, 65, 5171-5175

Lövgren-Bengtsson, K. (1998). Preparation and use of adjuvants. In *Methods in Microbiology. Volume 25. Immunology of Infection*. Edited by S.H.E. Kaufmann. & D. Kabelitz. Academic Press. 471-502.

Mahlandt, B. G., Klein, F., Lincoln, R. E., Haines, B. W., Jones, W. I., Jr. & Friedman, R. H. (1966). Immunologic studies of anthrax. IV. Evaluation of the immunogenicity of three components of anthrax toxin. *J. Immunol.*, 96, 727-733.

Mannhalter, J.W., Neychev, H.O., Zlabinger, G.J., Ahmad, R. & Eibl, M.M. (1985). Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake antigen presentation. *Clin. Exp. Immunol.*, 61, 143-151.

May K.R. & Harper G.J. (1957). The efficiency of various liquid impinger samplers in bacterial aerosols. *Brit. Ind. Med.*, 14: 287-297.

McBride, B.W., Dennis, M. & Farrar, G.H. (1993). SCID mice in biomedical research. *PHLS Microbiology Digest*, 10, 9-15.

McBride, B.W., Mogg, A., Telfer, J.L., Lever, M.S., Miller, J., Turnbull, P.C. B. & Baillie, L. (1998). Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. *Vaccine*, 16, 810-817.



McGovern, T.W., Christopher, G.W. & Eitzen, E.M. (1999). Cutaneous manifestations of biological warfare and related threat agents. *Arch. Dermatol.*, 135, 311-322.

Meselson, M., Guillemin, J., Hugh-Jones, M., Langmuir, A., Popova, I., Shelkov, A. & Yampolskaya, O. (1994). The Sverdlovsk anthrax outbreak of 1979. *Science*, 266, 1202-1208.

M'Fadyean, J. (1903). A further note with regard to the staining reaction of anthrax blood with methylene-blue. *J. Comp. Pathol.*, 16, 360-361.

Mikesell, P., Ivins, B.E., Ristoph, J.D. & Dreier, T. M. (1983). Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. *Infect. Immun.*, 39, 371 - 376.

Miller, J., McBride, B.W., Manchee, R.J., Moore, P. & Baillie, L.W.J. (1998a). Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis*. *Lett. App. Microbiol.*, 26, 56-60.

Miller, J., Petosa, C., Liddington, R.C. & Baillie, L.W.J. (1998b). Crystal structure of recombinant protective antigen and comparison with the wildtype structure. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 62.

Milne, J.C., Furlong, D., Hanna, P.C., Wall, J.S. & Collier, R.J. (1994). Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J. Biol.*

Nass, M. (1992). Anthrax epizootic in Zimbabwe, 1978-1980: due to deliberate spread ? *Physicians for Social Responsibility Quarterly*, 2, 198-209.

O'Brien, J., Friedlander, A., Dreier, T., Ezzell, J. & Leppla, S.H. (1985). Effects of anthrax toxin components on human neutrophils. *Infect. Immun.*, 47, 306-310.

Odenaal, M.W., Pieterse, P.M., De Vos, V. & Botha, A.D. (1991). The antibiotic sensitivity patterns of *Bacillus anthracis* isolated from the Kruger national park. *Onderstepoort. J. Vet. Res.*, 58, 17-19.

Pasteur, L., Chamberland, C. & Roux, E. (1881). De l'attenuation des virus et de leur retour de la virulence. Le vaccin du charbon. *C. R. Acad. Sci. (Paris)*, 92, 429-435. Cited by Bulloch, W. (1930). History of Bacteriology. In *A System of Bacteriology in relation to Medicine*. Volume 1. Editors P. Fildes. & J.C.G. Ledingham. Assisted by A. Harden. HMSO, Chapter 1, 81.

Pearson, H.E. (1970). Human infections caused by organisms of the *Bacillus* species. *Am. J. Clin. Pathol.*, 53, 506-515.

Percival, D.A., Frith, N.J. & Williamson, E.D. (1990). Manipulation of the immune response to the protective antigen of *Bacillus anthracis* by anti-idiotypic antibody. *Salisbury Medical Bulletin (Special Supplement)*, 68, 78-79.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. & Liddington, R.C. (1997).

Crystal structure of anthrax toxin protective antigen. *Nature*, 385, 833-838.

Pezard, C., Berche, P. & Mock, M. (1991). Contribution of individual toxin components to virulence of *Bacillus anthracis*. *Infect. Immun.*, 59, 3472-3477.

Pezard, C., Weber, M., Sirard, J-C., Berche, P. & Mock, M. (1995). Protective immunity induced by *Bacillus anthracis* toxin-deficient strains. *Infect. Immun.*, 63, 1369-1372.

Pfisterer, R.M. (1990). Retrospective verification of the diagnosis of anthrax by means of the intracutaneous skin test with the russian allergen "Anthraxin" in a recent epidemic in Switzerland. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 80.

Pitt, M.L.M., Ivins, B.E., Estep, J.E., Farchaus, J. & Friedlander, A.M. (1996). Comparison of the efficacy of purified protective antigen and MDPH to protect non-human primates from inhalation anthrax. *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 130.

Pitt, M.L.M., Little, S.F., Ivins, B.E., Fellows, P., Boles, J., Barth, J. Hewetson, J. & Friedlander, A.M. (1998). *In vitro* correlate of immunity in an animal model of inhalational anthrax. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 32.

Plotkin, S.A., Brachman, P.S., Utell, M., Bumford, F.H. & Atchison, M.M. (1960). An epidemic of inhalation anthrax, the first in the twentieth century. I. Clinical

features. *Am. J. Med*, 29, 992-1001.

Puziss, M. & Wright, G.G. (1963). Studies on immunity in anthrax X. Gel-adsorbed protective antigen for immunisation in man. *J. Bact.*, 85, 230.

Quinn, C.P., Turnbull, P.C.B. and Melling, J. (1990). Spore protein antigens specific to *Bacillus anthracis*. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 81-84.

Ramanathan, V.D., Badenoch-Jones, P. & Turk, J.L. (1979). Complement activation by aluminium and zirconium compounds. *Immunology*, 37, 881-888.

Redmond, C., Hall, G.A., Green, M. & Turnbull, P.C. B. (1996). Bacteriology, serology and pathology of experimental anthrax in pigs. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 79 - 82.

Redmond, C., Hall, G.A., Turnbull, P.C. B. & Gillgan, J.S. (1997). Experimentally assessed public health risks associated with pigs from farms experiencing anthrax. *Vet. Record*, 6 September, 244-247.

Redmond, C., Pearce, M.J., Manchee, R.J. & Berdal, B.P. (1998). Deadly relic of the great war. *Nature*, 393, 747-748.

Reed, L.J. & Muench, H.A. (1938). Simple method for estimating fifty per cent endpoints. *Am. J. Hyg.*, 27, 493-

Rich, V. (1980). Incident at military village No.19. *Nature*, 284, 294.

Rimmington, A. (1994). Russia's military microbiologists record victory against anthrax, but at what civilian cost ? *Microbiology Europe*, 2, 12-13.

Roberts, A.D., Belisle, J.T., Cooper, A.M. & Orme, I.M. (1998). Murine models of Tuberculosis. In *Methods in Microbiology. Volume 25. Immunology of Infection*. Edited by S.H.E. Kaufmann. & D. Kabelitz. Academic Press. 389-417.

Roitt, I. (1991). *Essential Immunology*. 7th Edition. Blackwell Scientific Publications.

Rosebury, T. & Kabat, E. A. (1947). Bacterial warfare: a critical analysis of the available agents, their possible military applications, and the means for protection against them. *J. Immunol.* 7-96.

Ross, J.M. (1957). The pathogenesis of anthrax following the administration of spores by the respiratory route. *J. Pathol. Bacteriol.*, 73, 485-494.

Rudbach, J.A., Johnson, D.A. & Ulrich, J.T. (1995). Ribi adjuvants: chemistry, biology and utility in vaccines for human and veterinary medicine. In *The Theory and Practical Application of Adjuvants*. Edited by D.E.S. Stewart-Tull. John Wiley & Sons, Chichester. Chapter 13, 287-313.

Russell, P., Eley, S.M., Green, M., Stagg, A., Taylor, R.R., Nelson, M., Beedham,

R.J., Bell, D.L., Rogers, D., Whittington, D. & Titball, R. (1998). Efficacy of doxycycline and ciprofloxacin against experimental *Yersinia pestis* infection. *J. Antimicro. Chemo.* 41, 301-305.

Salmon, D.E. (1896). *US Department of Agriculture, Bureau of Animal Industry, Special Report on Diseases of the Horse*. Government Printing Office, Washington, DC, 526-530.

Shlyakhov, E.N. & Shroit, I.G. (1964). Anthrax. Biological and immunological principles of diagnosis and prevention. Communication 2. Pathomorphological changes in skin in the anthraxin allergy test. *J. Hyg. Epidemiol. Microbiol. Immunol.*, 8, 307-312.

Shlyakhov, E.N. (1971). Anthrax. Biological and immunological principles of diagnosis and prevention. Communication 5. Correlation of anthraxin test indices with specific protection of guinea pigs. *J. Hyg. Epidemiol. Microbiol. Immunol.*, 15, 207-211.

Shlyakhov, E.N., Shvarts, S.A. & Gruz, E.V. (1973). Anthrax. Biological and immunological principles of diagnosis and prevention. Communication 7. Diagnosis of anthrax by means of anthraxin. *J. Hyg. Epidemiol. Microbiol. Immunol.*, 17, 279-284.

Shlyakhov, E.N. & Rubenstein, E. (1994). Human live anthrax vaccine in the former USSR. *Vaccine*, 12, 727-730.

- Shlyakhov, E. (1996). Anthraxin - a skin test for early and retrospective diagnosis of anthrax and anthrax vaccination assessment. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin Special Supplement*. 87; 109-110.
- Shlyakhov, E.N. & Rubenstein, E. (1996). Evaluation of the anthraxin skin test for diagnosis of acute and past human anthrax. *Eur. J. Clin. Microbiol. Infect. Dis.*, 15, 242-245.
- Shlyakhov, E.N., Rubenstein, E. & Novikov, I. (1997). Anthrax post-vaccinal cell-mediated immunity in humans: kinetics pattern. *Vaccine*, 15, 631-636.
- Shlyakhov, E. (1998). Anthraxin: confrontation with live and acellular anthrax vaccines. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, P41, 77.
- Slater, C. & Spitta, E.J. (1898). *An Atlas of Bacteriology*. The Scientific Press Limited London. 24.
- Smith, H. (1964). Microbial behaviour in natural and artificial environments. In *Microbial behaviour, 'In Vivo' and 'In Vitro'. 14<sup>th</sup> Symposium of the Society for General Microbiology, April 1964*. Edited by H. Smith & J. Taylor. Cambridge University Press. 1-29.
- Smith, H. (1995). The state and future of studies on bacterial pathogenicity. In *Virulence Mechanisms of Bacterial Pathogens*. Edited by J.A. Roth, C.A. Bolin, K.A. Brogden, F.C. Minion & M.J. Wannemuehler. ASM Press, Washington D.C.

Smith, H. & Keppie, J. (1954). Observations on experimental anthrax: Demonstration of a specific lethal factor produced *in vivo* by *Bacillus anthracis*. *Nature*, 173, 869-870.

Spencer, R.C. & Wilcox, M.H. (1993) Agents of biological warfare. *Rev. Med. Microbiol.*, 4, 138-143.

Stanley, J.L. & Smith, H. (1961). Purification of Factor I and recognition of a third factor of the anthrax toxin. *J. Gen. Micro.*, 26, 49-66.

Stepanov, A.S. & Leppla, S.H. (1996). Macrophages are killed by the plasmid- and chromosomally-encoded factors synthesized by *Bacillus anthracis* inside and outside the host cell. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 89.

Stepanov, A.S., Klimpel, K.R. & Leppla, S.H. (1996a). Extracellular proteases in *Bacillus anthracis*. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 87.

Stepanov, A.S., Mikshis, N.I. & Bolotnikova, M.F. (1996b). Role of chromosomally-encoded factors in virulence of *Bacillus anthracis* for mice and guinea pigs. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 86-87.



Sterne, M. (1937) The effects of different carbon dioxide concentrations on the growth of virulent anthrax strains. Pathogenicity and immunity tests on guinea pigs and sheep with anthrax variants derived from virulent strains. *Onderstepoort J. Vet. Sci. Anim. Ind.*, 9, 49-67.

Sterne, M. (1939). The use of anthrax vaccines prepared from avirulent (uncapsulated) variants of *Bacillus anthracis*. *Onderstepoort J. Vet. Sci. Anim. Ind.*, 13, 307-312.

Sterne, M. (1959). Anthrax. In *Infectious Diseases Of Animals. Diseases Due To Bacteria*. Edited by A.W. Stableforth & I.A. Galloway, Butterworths Scientific Publications, Volume 1, Chapter 2, 16-52.

Surcel, H.M., Troye-Blomberg, M., Paulie, S., Andersson, G., Moreno, C., Pasvol, G. & Ivanyi, J. (1994). Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. *Immunology*, 81, 171-176.

Tigertt, W.D. (1980). Anthrax. William Smith Greenfield M.D, F.R.C.P, professor superintendent, the Brown Animal Sanctuary Institution (1878-1881). Concerning the priority due to him for the production of the first vaccine against anthrax. *J. Hygiene (Cambs)*. 85, 415-420.

Tomcsik & Ivanovics (1938). *Zettschr. F. Immunitatsf*, 93, 196. Cited by Sterne (1959).

Topley, W.W.C. & Wilson, G.S. (1931). Anthrax. In *The Principles of Bacteriology and Immunity*. Edited By W.W.C. Topley. & G.S. Wilson. Edward Arnold & Co, London. Chapter LXXIV, 1129-1142.

Tresselt, H.B. & Boor, A.K. (1955). An antigen prepared *in vitro* effective for immunization against anthrax. III. Immunization of monkeys against anthrax. *J. Infect. Dis.*, 97, 207- 210.

Tuazon, C.U., Murray, H.W., Levy, C., Solny, M.N., Curtin, J.A. & Sheagren, J.N. (1979). Serious infections from *Bacillus* sp. *J. A. M. A.*, 241, 1137-1140.

Turell, M.J. & Knudson, G.B. (1987). Mechanical transmission of *Bacillus anthracis* by stable flies (*Stomoxys calcitrans*) and mosquitoes (*Aedes aegypti* and *Aedes taeniorhynchus*). *Infect. Immun.*, 55, 1859-1861.

Turnbull, P.C., Broster, M.G., Carman, J.A., Manchee, R.J. & Melling, J. (1986). Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Infect. Immun.*, 52, 356-363

Turnbull, P. C. B., Leppla, S.H., Broster, M. G., Quinn, C.P. & Melling, J. (1988). Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Med. Microbiol. Immunol.* 177, 293 - 303.

Turnbull, P.C.B., Stuart, F.A., Barrett, N.J. & Melling, J. (1990a). Anthrax in the UK. In *Proceedings of the International Workshop on Anthrax*. Salisbury Medical

Turnbull, P.C.B., Quinn, C.P., Hewson, R., Stockbridge, M.C. & Melling, J. (1990b). Protection conferred by microbially-supplemented UK and purified PA vaccines. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 89-91.

Turnbull, P. C. B. (1991). Anthrax vaccines: past, present and future. *Vaccine*, 9, 533 - 539.

Turnbull, P.C.B. (1992). Anthrax is alive and well. *PHLS Microbiology Digest*, 9, 103-106.

Turnbull, P.C.B., Bohm, R., Chizyuka, H. G. B., Fujikura, T., Hugh-Jones, M.E. & Melling, J. (1993). *Guidelines for the Surveillance and Control of Anthrax in Humans and Animals*. World Health Organisation, Geneva. WHO/Zoon/93.170.

Turnbull, P.C.B., Bowen, J.E., Gillgan, J.S. & Barrett, N.J. (1996). Incidence of anthrax, and environmental detection of *Bacillus anthracis* in the UK. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 5.

Turnbull, P.C.B., Bohm, R., Cosivi, O., Doganay, M., Hugh-Jones, M.E., Joshi, D.D., Lalitha, M.K. & de Vos, V. (1998). *Guidelines for the Surveillance and Control of Anthrax in Humans and Animals*. World Health Organisation, Geneva. WHO/EMC/ZDI./98.6. Third Edition.

- Turner, A.J., Galvin, J.W., Miller, G.T. & Rubira, R.J. (1998). Anthrax explodes in an Australian summer. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 3.
- Uchida, I., Sekizaki, T., Hashimoto, K. & Terakado, N. (1985). Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. *J. Gen. Micro.*, 131, 363-367.
- Vančúrik, J. (1966). Causes of the failure of antibiotic prophylaxis of inhalation anthrax and clearance of the spores from the lungs. *Folia Microbiologica*, 11, 459-464.
- Van den Bosch, C. (1996). Recalling an outbreak of gastrointestinal anthrax in northern Kenya, 1996. In *Proceedings of the International Workshop on Anthrax*. *Salisbury Medical Bulletin Special Supplement*. 87; 139.
- Vick, J.A., Lincoln, R.E., Klein, F., Mahlandt, B.G., Walker, J.S. & Fish, D.C. (1968). Neurological and physiological responses of the primate to anthrax toxin. *J. Infect. Dis*, 118, 85-103.
- Viney, J.L. (1994). Transgenic and knockout models for studying diseases of the immune system. *Curr. Opin. Genet. Dev.*, 4, 461-465.
- Wade, N. (1980) Death at Sverdlovsk: a critical diagnosis. *Science*, 209, 1501-1502.

- Walls, R.S. (1977). Eosinophil response to alum adjuvants: involvement of T-cells in non-antigen dependent mechanisms. *Proc. Soc. Exp. Biol. Med.*, 156, 431-435.
- Ward, M.K., McGann, V.G., Hogge, A.L., Huff, M.L., Kanode, R.G. & Roberts, E.O. (1965). Studies on anthrax infections in immunized guinea pigs. *J. Infect. Dis.*, 115, 59-67.
- Watson, D.W., Cromartie, W.J., Bloom, W.L., Kegeles, G. & Heckly, R.J. (1947). Studies on infection with *Bacillus anthracis*. III. Chemical and immunological properties of the protective antigen in crude extracts of skin lesions of *Bacillus anthracis*. *J. Infect. Dis.*, 80, 28-40.
- Welkos, S.L., Keener, T.J. & Gibbs, P.H. (1986). Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infect. Immun.*, 51, 795-800.
- Welkos, S.L. & Friedlander, A.M. (1988). Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. *Microb. Pathog.*, 5, 127-139.
- Welkos, S.L., Becker, D., Friedlander, A.M. & Trotter, R. (1990). Pathogenesis and host resistance to *Bacillus anthracis*: a mouse model. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 49-52.
- Welkos, S.L. (1991) Plasmid-associated virulence factors of non-toxigenic (pX01) *Bacillus anthracis*. *Microb. Pathog.*, 10, 183-198.

Welkos, S.L., Vietri, N.J. & Gibbs, P.H. (1993). Non-toxigenic derivatives of the Ames strain of *Bacillus anthracis* are fully virulent for mice: role of plasmid pX02 and chromosome in strain-dependent virulence. *Microb. Pathog*, 14, 381-388.

Williamson, E.D., Percival, D.A., Frith, N.J. & Kelly, D.C. (1990). Cell-mediated immune responses to the toxins of anthrax. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 92-94.

Williamson, E.D., Eley, S.M., Stagg, A.J., Green, M., Russell, P. & Titball, R.W. (1997). A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunised animals against pneumonic plague. *Vaccine*, 15, 1079-1084.

Wilson, G. (1979). The Brown Animal Sanatory Institution. *J. Hyg*, 82, 337-352.

Wilson, J.B. & Russell, K.E. (1964). Isolation of *Bacillus anthracis* from soil stored 60 years. *J. Bact*, 87, 237-238.

Woodall, J. (1998). Anthrax, human - Kazakhstan (Almaty). *Program for Monitoring Emerging Disease*, 15 July.

World Health Organisation. (1970). *Health Aspects of Chemical and Biological Weapons*. WHO, Geneva.

Wright, G. G., Hedberg, M.A. & Slein, J.B. (1954). Studies on immunity in anthrax

III. Elaboration of protective antigen in chemically-defined non-protein medium. *J.*

*Immunol*, 72, 263.

Wright, G. G. & Mandell, G.L. (1986). Anthrax toxin blocks priming of neutrophils by lipopolysaccharide and by muramyl dipeptide. *J. Exp. Med.*, 164, 1700-1709.

Young, G.A., Zelle, M.R. & Lincoln, R.E. (1946). Respiratory pathogenesis of *Bacillus anthracis* spores. I. methods of study and observation of pathogenesis. *J. Infect. Dis.*, 79, 233-246.

Zwartouw, H. T. & Smith, H. (1956). Polyglutamic acid from *Bacillus anthracis* grown *in vivo*, structure and aggressin activity. *Biochem. J.*, 63, 437-447.

Lightfoot, N.F., Scott, R.J.D. & Turnbull, P.C.B. (1990). Antimicrobial susceptibility of *Bacillus anthracis*. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 95-98.

Lincoln, R.E., Klein, F., Walker, J.S., Haines, B.W., Jones, W.I., Mahlandt, B.G. & Friedman, R.H. (1965). Successful treatment of Rhesus monkeys for septicemic anthrax. *Antimicrob. Agents Chemo.*, 759-763.

Lincoln, R. E., Walker, J. S., Klein, F., Rosenwald, A. J. & Jones, W. I., Jr. (1967). Value of field data for extrapolation in anthrax. *Fed. Proc.*, 26, 1558-1562.

Lincoln, R.E. & Fish, D.C. (1970). Anthrax toxin. In *Microbial Toxins III*, 1st Edition. Edited by T.C. Montie, S. Kadis. & S.J. Ajl. Academic Press, New York. Chapter 9, 361-414.

Little, S.F. & Knudson, G.B. (1986). Comparative efficacy of *Bacillus anthracis* live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. *Infect. Immun.*, 52, 509-512.

Little, S.F., Novak, J.M., Lowe, J.R., Leppla, S.H., Singh, Y., Klimpel, K.R., Lidgerding, B.C. & Friedlander, A.M. (1996). Characterisation of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. *Microbiology*, 142, 707-715.

Little, S.F., Ivins, B.E., Fellows, P.F. & Friedlander, A.M. (1997). Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea



Lövgren-Bengtsson, K. (1998). Preparation and use of adjuvants. In *Methods in Microbiology. Volume 25. Immunology of Infection*. Edited by S.H.E. Kaufmann. & D. Kabelitz. Academic Press. 471-502.

Mahlandt, B. G., Klein, F., Lincoln, R. E., Haines, B. W., Jones, W. I., Jr. & Friedman, R. H. (1966). Immunologic studies of anthrax. IV. Evaluation of the immunogenicity of three components of anthrax toxin. *J. Immunol.*, 96, 727-733.

Mannhalter, J.W., Neychev, H.O., Zlabinger, G.J., Ahmad, R. & Eibl, M.M. (1985). Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake antigen presentation. *Clin. Exp. Immunol.*, 61, 143-151.

May K.R. & Harper G.J. (1957). The efficiency of various liquid impinger samplers in bacterial aerosols. *Brit. Ind. Med.*, 14: 287-297.

McBride, B.W., Dennis, M. & Farrar, G.H. (1993). SCID mice in biomedical research. *PHLS Microbiology Digest*, 10, 9-15.

McBride, B.W., Mogg, A., Telfer, J.L., Lever, M.S., Miller, J., Turnbull, P.C. B. & Baillie, L. (1998). Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. *Vaccine*, 16, 810-817.

McGovern, T.W., Christopher, G.W. & Eitzen, E.M. (1999). Cutaneous manifestations of biological warfare and related threat agents. *Arch. Dermatol.*, 135, 311-322.

Meselson, M., Guillemin, J., Hugh-Jones, M., Langmuir, A., Popova, I., Shelkov, A. & Yampolskaya, O. (1994). The Sverdlovsk anthrax outbreak of 1979. *Science*, 266, 1202-1208.

M'Fadyean, J. (1903). A further note with regard to the staining reaction of anthrax blood with methylene-blue. *J. Comp. Pathol.*, 16, 360-361.

Mikesell, P., Ivins, B.E., Ristoph, J.D. & Dreier, T. M. (1983). Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. *Infect. Immun.*, 39, 371 - 376.

Miller, J., McBride, B.W., Manchee, R.J., Moore, P. & Baillie, L.W.J. (1998a). Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis*. *Lett. App. Microbiol.*, 26, 56-60.

Miller, J., Petosa, C., Liddington, R.C. & Baillie, L.W.J. (1998b). Crystal structure of recombinant protective antigen and comparison with the wildtype structure. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 62.

Milne, J.C., Furlong, D., Hanna, P.C., Wall, J.S. & Collier, R.J. (1994). Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J. Biol.*

Nass, M. (1992). Anthrax epizootic in Zimbabwe, 1978-1980: due to deliberate spread ? *Physicians for Social Responsibility Quarterly*, 2, 198-209.

O'Brien, J., Friedlander, A., Dreier, T., Ezzell, J. & Leppla, S.H. (1985). Effects of anthrax toxin components on human neutrophils. *Infect. Immun.*, 47, 306-310.

Odenaal, M.W., Pieterse, P.M., De Vos, V. & Botha, A.D. (1991). The antibiotic sensitivity patterns of *Bacillus anthracis* isolated from the Kruger national park. *Onderstepoort. J. Vet. Res.*, 58, 17-19.

Pasteur, L., Chamberland, C. & Roux, E. (1881). De l'attenuation des virus et de leur retour de la virulence. Le vaccin du charbon. *C. R. Acad. Sci. (Paris)*, 92, 429-435. Cited by Bulloch, W. (1930). History of Bacteriology. In *A System of Bacteriology in relation to Medicine*. Volume 1. Editors P. Fildes. & J.C.G. Ledingham. Assisted by A. Harden. HMSO, Chapter 1, 81.

Pearson, H.E. (1970). Human infections caused by organisms of the *Bacillus* species. *Am. J. Clin. Pathol.*, 53, 506-515.

Percival, D.A., Frith, N.J. & Williamson, E.D. (1990). Manipulation of the immune response to the protective antigen of *Bacillus anthracis* by anti-idiotypic antibody. *Salisbury Medical Bulletin (Special Supplement)*, 68, 78-79.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. & Liddington, R.C. (1997).

Crystal structure of anthrax toxin protective antigen. *Nature*, 385, 833-838.

Pezard, C., Berche, P. & Mock, M. (1991). Contribution of individual toxin components to virulence of *Bacillus anthracis*. *Infect. Immun.*, 59, 3472-3477.

Pezard, C., Weber, M., Sirard, J-C., Berche, P. & Mock, M. (1995). Protective immunity induced by *Bacillus anthracis* toxin-deficient strains. *Infect. Immun.*, 63, 1369-1372.

Pfisterer, R.M. (1990). Retrospective verification of the diagnosis of anthrax by means of the intracutaneous skin test with the russian allergen "Anthraxin" in a recent epidemic in Switzerland. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 80.

Pitt, M.L.M., Ivins, B.E., Estep, J.E., Farchaus, J. & Friedlander, A.M. (1996). Comparison of the efficacy of purified protective antigen and MDPH to protect non-human primates from inhalation anthrax. *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 130.

Pitt, M.L.M., Little, S.F., Ivins, B.E., Fellows, P., Boles, J., Barth, J. Hewetson, J. & Friedlander, A.M. (1998). *In vitro* correlate of immunity in an animal model of inhalational anthrax. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 32.

Plotkin, S.A., Brachman, P.S., Utell, M., Bumford, F.H. & Atchison, M.M. (1960). An epidemic of inhalation anthrax, the first in the twentieth century. I. Clinical

features. *Am. J. Med*, 29, 992-1001.

Puziss, M. & Wright, G.G. (1963). Studies on immunity in anthrax X. Gel-adsorbed protective antigen for immunisation in man. *J. Bact.*, 85, 230.

Quinn, C.P., Turnbull, P.C.B. and Melling, J. (1990). Spore protein antigens specific to *Bacillus anthracis*. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 81-84.

Ramanathan, V.D., Badenoch-Jones, P. & Turk, J.L. (1979). Complement activation by aluminium and zirconium compounds. *Immunology*, 37, 881-888.

Redmond, C., Hall, G.A., Green, M. & Turnbull, P.C. B. (1996). Bacteriology, serology and pathology of experimental anthrax in pigs. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 79 - 82.

Redmond, C., Hall, G.A., Turnbull, P.C. B. & Gillgan, J.S. (1997). Experimentally assessed public health risks associated with pigs from farms experiencing anthrax. *Vet. Record*, 6 September, 244-247.

Redmond, C., Pearce, M.J., Manchee, R.J. & Berdal, B.P. (1998). Deadly relic of the great war. *Nature*, 393, 747-748.

Reed, L.J. & Muench, H.A. (1938). Simple method for estimating fifty per cent endpoints. *Am. J. Hyg.*, 27, 493-

Rich, V. (1980). Incident at military village No.19. *Nature*, 284, 294.

Rimmington, A. (1994). Russia's military microbiologists record victory against anthrax, but at what civilian cost ? *Microbiology Europe*, 2, 12-13.

Roberts, A.D., Belisle, J.T., Cooper, A.M. & Orme, I.M. (1998). Murine models of Tuberculosis. In *Methods in Microbiology. Volume 25. Immunology of Infection*. Edited by S.H.E. Kaufmann. & D. Kabelitz. Academic Press. 389-417.

Roitt, I. (1991). *Essential Immunology*. 7th Edition. Blackwell Scientific Publications.

Rosebury, T. & Kabat, E. A. (1947). Bacterial warfare: a critical analysis of the available agents, their possible military applications, and the means for protection against them. *J. Immunol.* 7-96.

Ross, J.M. (1957). The pathogenesis of anthrax following the administration of spores by the respiratory route. *J. Pathol. Bacteriol.*, 73, 485-494.

Rudbach, J.A., Johnson, D.A. & Ulrich, J.T. (1995). Ribi adjuvants: chemistry, biology and utility in vaccines for human and veterinary medicine. In *The Theory and Practical Application of Adjuvants*. Edited by D.E.S. Stewart-Tull. John Wiley & Sons, Chichester. Chapter 13, 287-313.

Russell, P., Eley, S.M., Green, M., Stagg, A., Taylor, R.R., Nelson, M., Beedham,

R.J., Bell, D.L., Rogers, D., Whittington, D. & Titball, R. (1998). Efficacy of doxycycline and ciprofloxacin against experimental *Yersinia pestis* infection. *J. Antimicro. Chemo.* 41, 301-305.

Salmon, D.E. (1896). *US Department of Agriculture, Bureau of Animal Industry, Special Report on Diseases of the Horse*. Government Printing Office, Washington, DC, 526-530.

Shlyakhov, E.N. & Shroit, I.G. (1964). Anthrax. Biological and immunological principles of diagnosis and prevention. Communication 2. Pathomorphological changes in skin in the anthraxin allergy test. *J. Hyg. Epidemiol. Microbiol. Immunol.*, 8, 307-312.

Shlyakhov, E.N. (1971). Anthrax. Biological and immunological principles of diagnosis and prevention. Communication 5. Correlation of anthraxin test indices with specific protection of guinea pigs. *J. Hyg. Epidemiol. Microbiol. Immunol.*, 15, 207-211.

Shlyakhov, E.N., Shvarts, S.A. & Gruz, E.V. (1973). Anthrax. Biological and immunological principles of diagnosis and prevention. Communication 7. Diagnosis of anthrax by means of anthraxin. *J. Hyg. Epidemiol. Microbiol. Immunol.*, 17, 279-284.

Shlyakhov, E.N. & Rubenstein, E. (1994). Human live anthrax vaccine in the former USSR. *Vaccine*, 12, 727-730.

Shlyakhov, E. (1996). Anthraxin - a skin test for early and retrospective diagnosis of anthrax and anthrax vaccination assessment. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin Special Supplement*. 87; 109-110.

Shlyakhov, E.N. & Rubenstein, E. (1996). Evaluation of the anthraxin skin test for diagnosis of acute and past human anthrax. *Eur. J. Clin. Microbiol. Infect. Dis.*, 15, 242-245.

Shlyakhov, E.N., Rubenstein, E. & Novikov, I. (1997). Anthrax post-vaccinal cell-mediated immunity in humans: kinetics pattern. *Vaccine*, 15, 631-636.

Shlyakhov, E. (1998). Anthraxin: confrontation with live and acellular anthrax vaccines. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, P41, 77.

Slater, C. & Spitta, E.J. (1898). *An Atlas of Bacteriology*. The Scientific Press Limited London. 24.

Smith, H. (1964). Microbial behaviour in natural and artificial environments. In *Microbial behaviour, 'In Vivo' and 'In Vitro'. 14<sup>th</sup> Symposium of the Society for General Microbiology, April 1964*. Edited by H. Smith & J. Taylor. Cambridge University Press. 1-29.

Smith, H. (1995). The state and future of studies on bacterial pathogenicity. In *Virulence Mechanisms of Bacterial Pathogens*. Edited by J.A. Roth, C.A. Bolin, K.A. Brogden, F.C. Minion & M.J. Wannemuehler. ASM Press, Washington D.C.



Smith, H. & Keppie, J. (1954). Observations on experimental anthrax: Demonstration of a specific lethal factor produced *in vivo* by *Bacillus anthracis*. *Nature*, 173, 869-870.

Spencer, R.C. & Wilcox, M.H. (1993) Agents of biological warfare. *Rev. Med. Microbiol.*, 4, 138-143.

Stanley, J.L. & Smith, H. (1961). Purification of Factor I and recognition of a third factor of the anthrax toxin. *J. Gen. Micro.*, 26, 49-66.

Stepanov, A.S. & Leppla, S.H. (1996). Macrophages are killed by the plasmid- and chromosomally-encoded factors synthesized by *Bacillus anthracis* inside and outside the host cell. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 89.

Stepanov, A.S., Klimpel, K.R. & Leppla, S.H. (1996a). Extracellular proteases in *Bacillus anthracis*. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 87.

Stepanov, A.S., Mikshis, N.I. & Bolotnikova, M.F. (1996b). Role of chromosomally-encoded factors in virulence of *Bacillus anthracis* for mice and guinea pigs. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 86-87.

Sterne, M. (1937) The effects of different carbon dioxide concentrations on the growth of virulent anthrax strains. Pathogenicity and immunity tests on guinea pigs and sheep with anthrax variants derived from virulent strains. *Onderstepoort J. Vet. Sci. Anim. Ind.*, 9, 49-67.

Sterne, M. (1939). The use of anthrax vaccines prepared from avirulent (uncapsulated) variants of *Bacillus anthracis*. *Onderstepoort J. Vet. Sci. Anim. Ind.*, 13, 307-312.

Sterne, M. (1959). Anthrax. In *Infectious Diseases Of Animals. Diseases Due To Bacteria*. Edited by A.W. Stableforth & I.A. Galloway, Butterworths Scientific Publications, Volume 1, Chapter 2, 16-52.

Surcel, H.M., Troye-Blomberg, M., Paulie, S., Andersson, G., Moreno, C., Pasvol, G. & Ivanyi, J. (1994). Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. *Immunology*, 81, 171-176.

Tigertt, W.D. (1980). Anthrax. William Smith Greenfield M.D, F.R.C.P, professor superintendent, the Brown Animal Sanctuary Institution (1878-1881). Concerning the priority due to him for the production of the first vaccine against anthrax. *J. Hygiene (Cambs)*. 85, 415-420.

Tomcsik & Ivanovics (1938). *Zettschr. F. Immunitatsf*, 93, 196. Cited by Sterne (1959).

Topley, W.W.C. & Wilson, G.S. (1931). Anthrax. In *The Principles of Bacteriology and Immunity*. Edited By W.W.C. Topley. & G.S. Wilson. Edward Arnold & Co, London. Chapter LXXIV, 1129-1142.

Tresselt, H.B. & Boor, A.K. (1955). An antigen prepared *in vitro* effective for immunization against anthrax. III. Immunization of monkeys against anthrax. *J. Infect. Dis.*, 97, 207- 210.

Tuazon, C.U., Murray, H.W., Levy, C., Solny, M.N., Curtin, J.A. & Sheagren, J.N. (1979). Serious infections from *Bacillus* sp. *J. A. M. A.*, 241, 1137-1140.

Turell, M.J. & Knudson, G.B. (1987). Mechanical transmission of *Bacillus anthracis* by stable flies (*Stomoxys calcitrans*) and mosquitoes (*Aedes aegypti* and *Aedes taeniorhynchus*). *Infect. Immun.*, 55, 1859-1861.

Turnbull, P.C., Broster, M.G., Carman, J.A., Manchee, R.J. & Melling, J. (1986). Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Infect. Immun.*, 52, 356-363

Turnbull, P. C. B., Leppla, S.H., Broster, M. G., Quinn, C.P. & Melling, J. (1988). Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Med. Microbiol. Immunol.* 177, 293 - 303.

Turnbull, P.C.B., Stuart, F.A., Barrett, N.J. & Melling, J. (1990a). Anthrax in the UK. In *Proceedings of the International Workshop on Anthrax*. Salisbury Medical

Turnbull, P.C.B., Quinn, C.P., Hewson, R., Stockbridge, M.C. & Melling, J. (1990b). Protection conferred by microbially-supplemented UK and purified PA vaccines. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 89-91.

Turnbull, P. C. B. (1991). Anthrax vaccines: past, present and future. *Vaccine*, 9, 533 - 539.

Turnbull, P.C.B. (1992). Anthrax is alive and well. *PHLS Microbiology Digest*, 9, 103-106.

Turnbull, P.C.B., Bohm, R., Chizyuka, H. G. B., Fujikura, T., Hugh-Jones, M.E. & Melling, J. (1993). *Guidelines for the Surveillance and Control of Anthrax in Humans and Animals*. World Health Organisation, Geneva. WHO/Zoon/93.170.

Turnbull, P.C.B., Bowen, J.E., Gillgan, J.S. & Barrett, N.J. (1996). Incidence of anthrax, and environmental detection of *Bacillus anthracis* in the UK. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 87, 5.

Turnbull, P.C.B., Bohm, R., Cosivi, O., Doganay, M., Hugh-Jones, M.E., Joshi, D.D., Lalitha, M.K. & de Vos, V. (1998). *Guidelines for the Surveillance and Control of Anthrax in Humans and Animals*. World Health Organisation, Geneva. WHO/EMC/ZDI./98.6. Third Edition.

Turner, A.J., Galvin, J.W., Miller, G.T. & Rubira, R.J. (1998). Anthrax explodes in an Australian summer. *Abstracts of the 3rd International Conference on Anthrax*, University of Plymouth, 7-10 September 1998, 3.

Uchida, I., Sekizaki, T., Hashimoto, K. & Terakado, N. (1985). Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. *J. Gen. Micro.*, 131, 363-367.

Vančurík, J. (1966). Causes of the failure of antibiotic prophylaxis of inhalation anthrax and clearance of the spores from the lungs. *Folia Microbiologica*, 11, 459-464.

Van den Bosch, C. (1996). Recalling an outbreak of gastrointestinal anthrax in northern Kenya, 1996. In *Proceedings of the International Workshop on Anthrax*. *Salisbury Medical Bulletin Special Supplement*. 87; 139.

Vick, J.A., Lincoln, R.E., Klein, F., Mahlandt, B.G., Walker, J.S. & Fish, D.C. (1968). Neurological and physiological responses of the primate to anthrax toxin. *J. Infect. Dis*, 118, 85-103.

Viney, J.L. (1994). Transgenic and knockout models for studying diseases of the immune system. *Curr. Opin. Genet. Dev.*, 4, 461-465.

Wade, N. (1980) Death at Sverdlovsk: a critical diagnosis. *Science*, 209, 1501-1502.

- Walls, R.S. (1977). Eosinophil response to alum adjuvants: involvement of T-cells in non-antigen dependent mechanisms. *Proc. Soc. Exp. Biol. Med.*, 156, 431-435.
- Ward, M.K., McGann, V.G., Hogge, A.L., Huff, M.L., Kanode, R.G. & Roberts, E.O. (1965). Studies on anthrax infections in immunized guinea pigs. *J. Infect. Dis.*, 115, 59-67.
- Watson, D.W., Cromartie, W.J., Bloom, W.L., Kegeles, G. & Heckly, R.J. (1947). Studies on infection with *Bacillus anthracis*. III. Chemical and immunological properties of the protective antigen in crude extracts of skin lesions of *Bacillus anthracis*. *J. Infect. Dis.*, 80, 28-40.
- Welkos, S.L., Keener, T.J. & Gibbs, P.H. (1986). Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infect. Immun.*, 51, 795-800.
- Welkos, S.L. & Friedlander, A.M. (1988). Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. *Microb. Pathog.*, 5, 127-139.
- Welkos, S.L., Becker, D., Friedlander, A.M. & Trotter, R. (1990). Pathogenesis and host resistance to *Bacillus anthracis*: a mouse model. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 49-52.
- Welkos, S.L. (1991) Plasmid-associated virulence factors of non-toxigenic (pX01) *Bacillus anthracis*. *Microb. Pathog.*, 10, 183-198.

Welkos, S.L., Vietri, N.J. & Gibbs, P.H. (1993). Non-toxigenic derivatives of the Ames strain of *Bacillus anthracis* are fully virulent for mice: role of plasmid pX02 and chromosome in strain-dependent virulence. *Microb. Pathog*, 14, 381-388.

Williamson, E.D., Percival, D.A., Frith, N.J. & Kelly, D.C. (1990). Cell-mediated immune responses to the toxins of anthrax. In *Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin (Special Supplement)*, 68, 92-94.

Williamson, E.D., Eley, S.M., Stagg, A.J., Green, M., Russell, P. & Titball, R.W. (1997). A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunised animals against pneumonic plague. *Vaccine*, 15, 1079-1084.

Wilson, G. (1979). The Brown Animal Sanatory Institution. *J. Hyg*, 82, 337-352.

Wilson, J.B. & Russell, K.E. (1964). Isolation of *Bacillus anthracis* from soil stored 60 years. *J. Bact*, 87, 237-238.

Woodall, J. (1998). Anthrax, human - Kazakhstan (Almaty). *Program for Monitoring Emerging Disease*, 15 July.

World Health Organisation. (1970). *Health Aspects of Chemical and Biological Weapons*. WHO, Geneva.

Wright, G. G., Hedberg, M.A. & Slein, J.B. (1954). Studies on immunity in anthrax

III. Elaboration of protective antigen in chemically-defined non-protein medium. *J.*

*Immunol.*, 72, 263.

Wright, G. G. & Mandell, G.L. (1986). Anthrax toxin blocks priming of neutrophils by lipopolysaccharide and by muramyl dipeptide. *J. Exp. Med.*, 164, 1700-1709.

Young, G.A., Zelle, M.R. & Lincoln, R.E. (1946). Respiratory pathogenesis of *Bacillus anthracis* spores. I. methods of study and observation of pathogenesis. *J. Infect. Dis.*, 79, 233-246.

Zwartouw, H. T. & Smith, H. (1956). Polyglutamic acid from *Bacillus anthracis* grown *in vivo*, structure and aggressin activity. *Biochem. J.*, 63, 437-447.